PATENT COOPERATION TREATY

From the INTERNATIONAL BUREAU

To:

Assistant Commissioner for Patents
United States Patent and Trademark Office
Box PCT
Washington, D.C.20231
ETATS-UNIS D'AMERIQUE

in its capacity as elected Office

PCT

NOTIFICATION OF ELECTION

(PCT Rule 61.2)

Date of mailing (day/month/year) 19 October 2000 (19.10.00)	
International application No. PCT/GB00/00654	**Applicant's or agent's file reference** IS/BP5842158
International filing date (day/month/year) 24 February 2000 (24.02.00)	**Priority date** (day/month/year) 24 February 1999 (24.02.99)
Applicant CHEETHAM, Peter, Samuel, James et al	

1. The designated Office is hereby notified of its election made:

☒ in the demand filed with the International Preliminary Examining Authority on:

20 September 2000 (20.09.00)

☐ in a notice effecting later election filed with the International Bureau on:

2. The election ☒ was

☐ was not

made before the expiration of 19 months from the priority date or, where Rule 32 applies, within the time limit under Rule 32.2(b).

The International Bureau of WIPO **34, chemin des Colombettes** **1211 Geneva 20, Switzerland** Facsimile No.: (41-22) 740.14.35	Authorized officer Pascal Piriou Telephone No.: (41-22) 338.83.38

Form PCT/IB/331 (July 1992)

ENT COOPERATION TREATY

PCT

INTERNATIONAL SEARCH REPORT

(PCT Article 18 and Rules 43 and 44)

Applicant's or agent's file reference IS/BP5842158	**FOR FURTHER ACTION** see Notification of Transmittal of International Search Report (Form PCT/ISA/220) as well as, where applicable, item 5 below.	
International application No. PCT/GB 00/ 00654	International filing date *(day/month/year)* 24/02/2000	(Earliest) Priority Date *(day/month/year)* 24/02/1999
Applicant ZYLEPSIS LIMITED et al.		

This International Search Report has been prepared by this International Searching Authority and is transmitted to the applicant according to Article 18. A copy is being transmitted to the International Bureau.

This International Search Report consists of a total of 4 sheets.

☒ It is also accompanied by a copy of each prior art document cited in this report.

1. **Basis of the report**
   a. With regard to the **language**, the international search was carried out on the basis of the international application in the language in which it was filed, unless otherwise indicated under this item.
      - ☐ the international search was carried out on the basis of a translation of the international application furnished to this Authority (Rule 23.1(b)).

   b. With regard to any **nucleotide and/or amino acid sequence** disclosed in the international application, the international search was carried out on the basis of the sequence listing :
      - ☐ contained in the international application in written form.
      - ☐ filed together with the international application in computer readable form.
      - ☐ furnished subsequently to this Authority in written form.
      - ☐ furnished subsequently to this Authority in computer readble form.
      - ☐ the statement that the subsequently furnished written sequence listing does not go beyond the disclosure in the international application as filed has been furnished.
      - ☐ the statement that the information recorded in computer readable form is identical to the written sequence listing has been furnished

2. ☐ **Certain claims were found unsearchable** (See Box I).
3. ☐ **Unity of invention is lacking** (see Box II).
4. With regard to the **title**,
   - ☒ the text is approved as submitted by the applicant.
   - ☐ the text has been established by this Authority to read as follows:

5. With regard to the **abstract**,
   - ☒ the text is approved as submitted by the applicant.
   - ☐ the text has been established, according to Rule 38.2(b), by this Authority as it appears in Box III. The applicant may, within one month from the date of mailing of this international search report, submit comments to this Authority.
6. The figure of the **drawings** to be published with the abstract is Figure No. ______
   - ☐ as suggested by the applicant.
   - ☐ because the applicant failed to suggest a figure.
   - ☐ because this figure better characterizes the invention.
   - ☒ None of the figures.

INTERNATIONAL SEARCH REPORT

In[illegible]onal Application No
PCT/GB 00/00654

A. CLASSIFICATION OF SUBJECT MATTER

IPC 7 C12P7/22 C12P7/24 C12P7/42 C12P39/00 C07C47/58
C12N15/01 C12N1/14 C12N1/16 //(C12P7/22,C12R1:40,
C12R1:645,C12R1:68),(C12P7/24,C12R1:40,C12R1:645,C12R1:68),

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 7 C12P C07C C12N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X A	GB 2 301 103 A (DANISCO A/S) 27 November 1996 (1996-11-27) page 4 -page 10; claim 42	1-39, 51-63 40-50
X	FAULDS C B ET AL: "RELEASE OF FERULIC ACID FROM WHEAT BRAN BY A FERULIC ACID ESTERASE (FAE-III) FROM ASPERGILLUS NIGER" APPLIED MICROBIOLOGY AND BIOTECHNOLOGY,DE,SPRINGER VERLAG, BERLIN, vol. 43, no. 6, 1 January 1995 (1995-01-01), pages 1082-1087, XP000603403 ISSN: 0175-7598	1-39, 51-63
A	the whole document	40-50
X	EP 0 885 968 A (GIVAUDAN-ROURE (INT.) S.A.) 23 December 1998 (1998-12-23) the whole document	1-39

-/--

☒ Further documents are listed in the continuation of box C.

☒ Patent family members are listed in annex.

° Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search 6 June 2000	Date of mailing of the international search report 13/06/2000
Name and mailing address of the ISA European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Authorized officer Douschan, K

INTERNATIONAL SEARCH REPORT

In[illegible]onal Application No
PCT/GB 00/00654

A. CLASSIFICATION OF SUBJECT MATTER
IPC 7 (C12P7/42,C12R1:40,C12R1:645,C12R1:68)

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 94 13614 A (QUEST INT. B.V.) 23 June 1994 (1994-06-23) the whole document ---	1-39
X	WO 94 02621 A (V. MANE FILS S.A.) 3 February 1994 (1994-02-03) the whole document ---	1-39
X	WO 96 08576 A (I.N.R.A.) 21 March 1996 (1996-03-21) the whole document ---	1-39
X	EP 0 857 789 A (KYOWA HAKKO KOGYO CO., LTD.) 12 August 1998 (1998-08-12) claims 5-14 --- -/--	1-36

☒ Further documents are listed in the continuation of box C.

☒ Patent family members are listed in annex.

° Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search 6 June 2000	Date of mailing of the international search report
Name and mailing address of the ISA European Patent Office, P.B. 5818 Patentlaan 2 NL – 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Authorized officer Douschan, K

INTERNATIONAL SEARCH REPORT

In[illegible]onal Application No
PCT/GB 00/00654

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication,where appropriate, of the relevant passages	Relevant to claim No.
X	WO 96 39859 A (ZYLEPSIS LTD.) 19 December 1996 (1996-12-19) cited in the application example 10 -----	37-39

2

Form PCT/ISA/210 (continuation of second sheet) (July 1992)

INTERNATIONAL SEARCH REPORT

Info[illegible]n on patent family members

I[illegible]onal Application No
PCT/GB 00/00654

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
GB 2301103 A	27-11-1996	NONE	
EP 0885968 A	23-12-1998	AU 7196498 A	24-12-1998
		BR 9802011 A	08-02-2000
		CA 2238215 A	19-12-1998
		CZ 9801883 A	13-01-1999
		JP 11069990 A	16-03-1999
		PL 326882 A	21-12-1998
		ZA 9805146 A	21-12-1998
WO 9413614 A	23-06-1994	AU 5698794 A	04-07-1994
WO 9402621 A	03-02-1994	FR 2694020 A	28-01-1994
		AT 173296 T	15-11-1998
		DE 69322067 D	17-12-1998
		DE 69322067 T	15-07-1999
		EP 0606441 A	20-07-1994
		JP 7500253 T	12-01-1995
		US 5712132 A	27-01-1998
WO 9608576 A	21-03-1996	FR 2724394 A	15-03-1996
		AT 174632 T	15-01-1999
		DE 69506724 D	28-01-1999
		DE 69506724 T	01-07-1999
		EP 0781345 A	02-07-1997
		ES 2128074 T	01-05-1999
		US 5866380 A	02-02-1999
EP 0857789 A	12-08-1998	CA 2228816 A	07-08-1998
		CN 1206045 A	27-01-1999
		JP 10276788 A	20-10-1998
		US 5955137 A	21-09-1999
WO 9639859 A	19-12-1996	AU 2626895 A	04-01-1996
		AU 6008996 A	30-12-1996
		EP 0764147 A	26-03-1997
		EP 0831715 A	01-04-1998
		JP 11506339 T	08-06-1999
		JP 10501216 T	03-02-1998

CLAIMS:

1. A method of converting a first composition comprising ferulic acid into a second composition comprising vanillin, said method comprising treating said first composition with *Pseudomonas putida* under conditions such that ferulic acid is converted into vanillin, and the vanillin accumulates.

2. A method according to claim 1 wherein said strain is *Pseudomonas putida* IMI382568.

3. A method according to claim 1 or 2 wherein said strain is capable of producing both vanillic acid and vanillin from ferulic acid, the ratio thereof being pH-dependant; and wherein a pH is selected and maintained which relatively favours accumulation of vanillin.

4. A method of converting a first composition comprising ferulic acid into a composition comprising vanillin by (i) converting said first composition which comprises ferulic acid into a second composition which comprises vanillic acid by treatment with a microorganism selected from *Pseudomonas putida* and *Rhodotorula glutinis;* and (ii) treating said second composition with a second microorganism selected from *Aspergillus fumigatus* and *Micromucor isabellinus* for converting vanillic acid with vanillin.

5. A method according to claim 4 wherein said first microorganism is *Pseudomonas putida* NCIMB40988 or *Rhodotorula* glutinis IMI379896.

6. A method according to any preceding claim including a preliminary step of obtaining said first composition comprising ferulic acid from a plant material by a process comprising:

(a) treating the plant material to produce a solution containing a ferulic acid ester; and

(b) treating said solution with an enzyme composition having ferulic acid esterase activity under conditions such that ferulic acid esters are converted into ferulic acid.

7. A method according to claim 6 wherein said plant material is selected from maize, wheat, sugar beet and rice materials.

8. A method according to claim 7 wherein said plant material comprises fibre, bran or straw.

9. A method according to claim 6, 7 or 8 wherein in step (a) the plant material is treated with a solution containing citric acid.

10. A method according to claim 9 wherein said plant material is treated in the temperature range 50-250°.

11. A method according to claim 6 wherein the plant material comprises sugar beet fibre and step (a) involves heating in water.

12. A method according to any of claims 6 to 11 wherein step (b) employs an enzyme derived from a species of *Aspergillus* or *Humicola insolens*.

13. A method according to claim 10 wherein the enzyme is derived from *Humicola insolens* and treatment is effected substantially in the pH range 6-7.

14. A method of converting a first composition comprising a ferulic acid ester into a second composition comprising vanillic acid, said method comprising treating said first composition with one or more microorganisms selected from *Penicillium* and *Aspergillus* species possessing both ferulic acid esterase activity and intra-sidechain cleavage activity such that they are capable of converting ferulic acid glycosides into vanillic acid under conditions such that said ester is converted into vanillic acid.

15. A method according to claim 14 wherein said microorganism is selected from *P.chrysogenum, A.niger* and *A.flavus*.

16. A method according to claim 15 wherein said microorangism is selected from P.chrysogenum IMI379900, A.flavus IMI379895 and A.niger IMI379904.

17. A process according to any of claims 14 to 16 wherein said ferulic acid ester is provided in the form of plant material, said microorganism acting directly on said plant material.

18. A method according to any of claims 14 to 17 wherein said second composition is treated with one or more further microorganisms for converting said vanillic acid into vanillin.

19. A method according to claim 18 wherein said second composition is treated with *Aspergillus fumigatus* or *Micromucor isabellinus* for converting vanillic acid into vanillin.

20. A method according to claim 19 wherein said second composition is treated with *A.fumigatus* IMI379902 or *M.isabellinus* IMI379893.

21. A method according to any of claims 1 to 13 or 18 to 20 wherein said conversion into vanillin is effected in an aqueous phase which is contacted with an organic phase which extracts said at least one desired component.

22. A method according to claim 1 or claim 4 including a preliminary step of obtaining a strain of microorganism for use in the method of claim 1 or step (ii) of claim 4 comprising screening a multiplicity of colonies by means of a reagent suitable for detecting aldehydes.

23. A method according to claim 22 wherein the multiplicity of colonies are obtained by mutation.

24. *Pseudomonas putida* NCIMB40988 or a mutant thereof capable of converting ferulic acid into vanillic acid.

25. *Rhodotorula glutinis* IMI379896 or a mutant thereof capable of converting ferulic acid into vanillic acid.

26. *Penicillium chrysogenum* IMI379900 or a mutant thereof capable of converting a ferulic acid ester into vanillic acid.

27. *Aspergillus flavus* IMI379895 or a mutant thereof capable of converting a ferulic acid ester into vanillic acid.

28. *Aspergillus niger* IMI379904 or a mutant thereof capable of converting a ferulic acid ester into vanillic acid.

29. *Pseudomonas putida* IMI382568 or a mutant thereof capable of converting ferulic acid into vanillin.

30. *Aspergillus fumigatus* IMI379902 or a mutant thereof capable of converting vanillic acid into vanillin.

31. *Micromucor isabellinus* IMI379893 or a mutant thereof capable of converting vanillic acid into vanillin.

32. A genetically engineered organism which has been transformed with nucleic acid derived directly or indirectly from the strain defined in any of claims 24 to 31 and has thereby acquired the capability set out in that claim.

33. An extract or enzyme(s) of an organism defined in any of claims 24 to 32 possessing the activity specified for the organism.

[illegible]ATENT COOPERATION TR[illegible]Y

PCT

REC'D 14 JUN 2001
WIPO PCT

INTERNATIONAL PRELIMINARY EXAMINATION REPORT

(PCT Article 36 and Rule 70)

14

Applicant's or agent's file reference IS/BP5842158	**FOR FURTHER ACTION**	See Notification of Transmittal of International Preliminary Examination Report (Form PCT/IPEA/416)
International application No. PCT/GB00/00654	International filing date *(day/month/year)* 24/02/2000	Priority date *(day/month/year)* 24/02/1999

International Patent Classification (IPC) or national classification and IPC
C12P7/22

Applicant
ZYLEPSIS LIMITED et al.

1. This international preliminary examination report has been prepared by this International Preliminary Examining Authority and is transmitted to the applicant according to Article 36.

2. This REPORT consists of a total of 8 sheets, including this cover sheet.

   ☒ This report is also accompanied by ANNEXES, i.e. sheets of the description, claims and/or drawings which have been amended and are the basis for this report and/or sheets containing rectifications made before this Authority (see Rule 70.16 and Section 607 of the Administrative Instructions under the PCT).

   These annexes consist of a total of 5 sheets.

3. This report contains indications relating to the following items:

   I ☒ Basis of the report
   II ☐ Priority
   III ☐ Non-establishment of opinion with regard to novelty, inventive step and industrial applicability
   IV ☒ Lack of unity of invention
   V ☒ Reasoned statement under Article 35(2) with regard to novelty, inventive step or industrial applicability; citations and explanations suporting such statement
   VI ☐ Certain documents cited
   VII ☒ Certain defects in the international application
   VIII ☒ Certain observations on the international application

Date of submission of the demand 20/09/2000	Date of completion of this report 12.06.2001
Name and mailing address of the international preliminary examining authority: European Patent Office D-80298 Munich Tel. +49 89 2399 - 0 Tx: 523656 epmu d Fax: +49 89 2399 - 4465	Authorized officer Douschan, K Telephone No. +49 89 2399 8702

Form PCT/IPEA/409 (cover sheet) (January 1994)

INTERNATIONAL PRELIMINARY EXAMINATION REPORT

International application No. PCT/GB00/00654

I. Basis of the report

1. With regard to the **elements** of the international application *(Replacement sheets which have been furnished to the receiving Office in response to an invitation under Article 14 are referred to in this report as "originally filed" and are not annexed to this report since they do not contain amendments (Rules 70.16 and 70.17))*:

Description, pages:

1-47	as originally filed

Claims, No.:

1-33	as received on	14/05/2001	with letter of	14/05/2001

2. With regard to the **language**, all the elements marked above were available or furnished to this Authority in the language in which the international application was filed, unless otherwise indicated under this item.

These elements were available or furnished to this Authority in the following language: , which is:

☐ the language of a translation furnished for the purposes of the international search (under Rule 23.1(b)).
☐ the language of publication of the international application (under Rule 48.3(b)).
☐ the language of a translation furnished for the purposes of international preliminary examination (under Rule 55.2 and/or 55.3).

3. With regard to any **nucleotide and/or amino acid sequence** disclosed in the international application, the international preliminary examination was carried out on the basis of the sequence listing:

☐ contained in the international application in written form.
☐ filed together with the international application in computer readable form.
☐ furnished subsequently to this Authority in written form.
☐ furnished subsequently to this Authority in computer readable form.
☐ The statement that the subsequently furnished written sequence listing does not go beyond the disclosure in the international application as filed has been furnished.
☐ The statement that the information recorded in computer readable form is identical to the written sequence listing has been furnished.

4. The amendments have resulted in the cancellation of:

☐ the description, pages:
☐ the claims, Nos.:
☐ the drawings, sheets:

5. ☐ This report has been established as if (some of) the amendments had not been made, since they have been considered to go beyond the disclosure as filed (Rule 70.2(c)):

(Any replacement sheet containing such amendments must be referred to under item 1 and annexed to this report.)

6. Additional observations, if necessary:

IV. Lack of unity of invention

1. In response to the invitation to restrict or pay additional fees the applicant has:

   ☐ restricted the claims.

   ☐ paid additional fees.

   ☐ paid additional fees under protest.

   ☐ neither restricted nor paid additional fees.

2. ☒ This Authority found that the requirement of unity of invention is not complied and chose, according to Rule 68.1, not to invite the applicant to restrict or pay additional fees.

3. This Authority considers that the requirement of unity of invention in accordance with Rules 13.1, 13.2 and 13.3 is

   ☐ complied with.

   ☒ not complied with for the following reasons:
   see separate sheet

4. Consequently, the following parts of the international application were the subject of international preliminary examination in establishing this report:

   ☐ all parts.

   ☒ the parts relating to claims Nos. 1-33.

V. Reasoned statement under Article 35(2) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement

1. Statement

Novelty (N)	Yes:	Claims	1-13,16,19,20,22-33
	No:	Claims	14,15,17,18,21
Inventive step (IS)	Yes:	Claims	1-13,16,19,20,22-25,29-33
	No:	Claims	14,15,17,18,21,26-28
Industrial applicability (IA)	Yes:	Claims	1-33
	No:	Claims	

2. Citations and explanations
   see separate sheet

VII. Certain defects in the international application

The following defects in the form or contents of the international application have been noted:
see separate sheet

VIII. Certain observations on the international application

The following observations on the clarity of the claims, description, and drawings or on the question whether the claims are fully supported by the description, are made:
see separate sheet

Re Item IV

Lack of unity of invention

An objection to non-unity is according to Rule 13 PCT is raised. Nevertheless, since the international examination phase has a limited time, no payment of further fees is required at the moment, but an objection to non-unity will be raised in the national/regional phase before the EPO.

a) The filing of new claims during the international phase did not overcome the non-unity objection. **Three different inventions**, which are not so linked to form one single general inventive concept as required by Rule 13 PCT, have been identified.

Invention 1 (claims 1-3, 6-13, 21-23, 29, 32 and 33) solves the problem of providing a process for the preparation of vanillin, starting from ferulic acid. This problem has been solved by using a microorganism of the species *Pseudomonas putida*.

Invention 2 (claims 4-13, 21-25 and 30-33) concerns a process for the conversion of ferulic acid to vanillic acid using *Pseudomonas putida* or *Rhodotorula glutinis* and subsequent treatment with *Aspergillus fumigatus* or *Micromucor isabellinus to* yield vanillin. This means, even if the process of invention 2 is regarded as a multistep process according to invention one, the solution to the said problem according to invention 1 is solved in a different way in that different microorganisms are used.

Invention 3 (claims 14-21, 26-28, 32 and 33) solves the problem of finding a process for the production of vanillic acid from ferulic acid esters, whereby microorganisms of the genus *Penicillium* and *Aspergillus* are used. Therefore invention 3 concerns a different problem and solution than the other two inventions.

b) Consequently, the present patent application consists of 3 inventions, whereas the said inventions are not linked by one single general inventive concept since they concern different problems and/or solutions. Only if all microorganisms mentioned have a capability to convert ferulic acid into vanillin and/or vanillic acid **and** to convert vanillin acid into vanillin **and** have both ferulic acid esterase activity

(=FAE) and intra-side chain cleavage activity, unity of invention could be acknowledged. Nevertheless, this is, according to the present claims 1-33, not the case.

c) In order to fulfill the requirements for unity in accordance with Rule 13 PCT, an invention has to solve one single problem with one single solution or a group of solutions so linked as to form one single general inventive concept, when compared with the prior art. In the present case there are different features contributing to the prior art, so that the requirements for unity are not fulfilled. In the present case different compounds of different origin are transformed by the use of different microorganisms having different characteristics.

Re Item V

Reasoned statement under Article 35(2) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement

1. The documents mentioned in the International Search Report are cited by the following abbreviations:

D1: GB-A-2 301 103 (DANISCO A/S) 27 November 1996 (1996-11-27),
D2: FAULDS C B ET AL: 'RELEASE OF FERULIC ACID FROM WHEAT BRAN BY A FERULIC ACID ESTERASE (FAE-III) FROM ASPERGILLUS NIGER' APPLIED MICROBIOLOGY AND BIOTECHNOLOGY,DE,SPRINGER VERLAG, BERLIN, vol. 43, no. 6, 1 January 1995 (1995-01-01), pages 1082-1087,
D3: EP-A-0 885 968 (GIVAUDAN-ROURE (INT.) S.A.) 23 December 1998 (1998-12-23),
D4: WO 94 13614 A (QUEST INT. B.V.) 23 June 1994 (1994-06-23),
D5: WO 94 02621 A (V. MANE FILS S.A.) 3 February 1994 (1994-02-03),
D6: WO 96 08576 A (I.N.R.A.) 21 March 1996 (1996-03-21),
D7: EP-A-0 857 789 (KYOWA HAKKO KOGYO CO., LTD.) 12 August 1998 (1998-08-12), and
D8: WO 96 39859 A (ZYLEPSIS LTD.) 19 December 1996 (1996-12-19).

2. **Claims 1-13, 16, 19, 20, 22-25 and 29-33:**

The microorganisms mentioned in the said claims are neither disclosed in nor suggested by the prior art documents for use in the claimed processes. Therefore novelty and the presence of an inventive step can be acknowledged for the said claims.

3. **Claims 14, 15, 17, 18 and 21:**

D6 concerns a process for the preparation of vanillic acid starting from ferulic acid or its esters or glycosides (cf. p. 8), using microorganisms of the genus *Penicillium and Aspergillus*, which are specified as e.g. *A. niger* or *Penicillium verrucosum* (cf. p. 5).
Although the names and activities of the enzymes (FAE and intra side chain cleavage activity) are not specified in D6, since an identical microorganism is used it is assumed that it contains the same enzymes. The mere identification of the enzymes as done in the objected claims is not sufficient to establish novelty as long as a known microorganism falls within the scope of the claims. Since D6 discloses an identical process using a.o. *Aspergillus niger*, novelty, and consequently, the presence of an inventive step cannot be acknowledged for claims 14, 15, 17, 18 and 21.

4. **Claims 26-28:**

Although the specific, deposited microorganisms claimed in the said claims are not mentioned in D6, an inventive step can nevertheless not be acknowledged since microorganisms having the same activity (i.e. converting ferulic acid or its esters/glycosides into vanillic acid) of the same genus are known from D6. it is merely a matter of routine screening for suitable species when a genus is known. Since no comparative test data showing a surprising superior effect of the claimed microorganisms is present, an inventive step cannot be acknowledged.

Re Item VII

Certain defects in the international application

a) Contrary to the requirements of Rule 5.1(a)(ii) PCT, the relevant background art disclosed in the documents D1-D7 is not mentioned in the description, nor are these documents identified therein.

b) If the present application contains registered trademarks (see e.g. the term “Biolafitte“ on p. 19), they should be acknowledged as such.

Re Item VIII

Certain observations on the international application

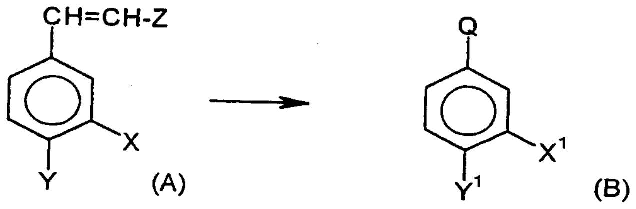
a) Contrary to the requirements of Art. 6 PCT, the description has not been adapted to the newly filed claims.

b) The reference in claim 13 to claim 10 appears to be wrong and should probably read "claim 12" (Art. 6 PCT).

c) In claim 10 the unit of the temperature value is missing - it is assumed that "°C" is meant (Art. 6 PCT).

CLAIMS:

1. A method of converting a first composition comprising one or more species of formula (A) into a second composition comprising one or more species of formula (B):



where X, Y, X' and Y' are independently selected from H, OH and OMe; Z is CO_2H, CO_2*carb* (where *carb* represents a carbohydrate residue), CHO or CH_2OH, and Q is CHO, CO_2H or CH_2OH, said method comprising treating said first composition with one or more microorganisms under conditions such that (A) is converted into (B); said microorganism(s) being selected, from (a) *Pseudomonas putida*; (b) *Rhodotorula* species and other yeasts capable of converted ferulic acid into vanillic acid;(c) microorganisms possessing both ferulic acid esterase activity and intra-sidechain cleavage activity such that they are capable of converting ferulic acid glycosides into vanillin and/or vanillic acid; and (d) *Micromucor isabellinus* or *Aspergillus fumigatus* strains capable of converting vanillin acid into vanillin.

2. A method according to claim 1 wherein Y and Y' are OH.

3. A method according to claim 1 or claim 2 wherein Z is CO_2H or CO_2*carb*.

4. A method according to claim 1, 2 or 3 wherein said first composition comprises ferulic acid or one or more ferulic acid esters and said second composition comprises vanillic acid and/or vanillyl alcohol and/or vanillin.

5. A method according to Claim 4 wherein said first composition comprises ferulic acid and said second composition comprises vanillic acid and said microorganism is selected from (a) and (b)

6. A method accroding to Claim 5 wherein said microorganism is *Rhodotorula glutinis*.

7. A method according to Claim 5 wherein said microorganism is *Pseudomonas putida* NCIMB40988 or *Rhodotorula glutinis* IMI379896.

8. A method according to Claim 4 wherein said first composition comprises a ferulic acid ester and said microorganism is selected from (c).

9. A method according to Claim 8 wherein said microorganism is selected from *Penicillium* and *Aspergillus* species.

10. A method according to Claim 9 wherein said microorganism is selected from *P.chrysogenum, A.niger* and *A.flavus.*

11. A method according to Claim 10 wherein said microorganism is selected from P.chrysogenum IMI379901, A.flavus IMI379895 and A.niger IMI379904.

12. A process according to any of claims 8 to 11 wherein said ferulic acid ester is provided in the form of plant material, said microorganism acting directly on said plant material.

13. A method according to any preceding Claim wherein said treatment of the first composition affords a second composition comprising vanillic acid, and said second composition is treated with one or more further microorganisms for converting said vanillic acid into vanillin.

14. A method according to Claim 13 wherein said second composition is treated with a second microorganism for converting said vanillic acid into vanillyl alcohol; and then with a third microorganism for converting said vanillyl alcohol into vanillin.

15. A method according to Claim 14 wherein said second microorganism is *Zygorhynchus moelleri.*

16. A method according to Claim 14 wherein said second micoorganism is *Zygorhynchus moelleri* IMI379899.

17. A method according to Claim 14, 15 or 16 wherein said third microorganism in *Brevundimonas vesicularis*.

18. A method according to Claim 17 wherein said third microorganism in *Brevundimonas vesicularis* NCIMB40987.

19. A method according to Claim 11 wherein said second composition is treated with *Aspergillus fumigatus* or *Micromucor isabellinus* for converting vanillic acid into vanillin.

20. A method according to Claim 17 wherein said second composition is treated with *A.fumigatus* IMI379902 or *M.isabellinus* IMI379893.

21. A method according to Claim 4 wherein said first composition is treated with a strain of *Pseudomonas* under conditions such that vanillin accumulates.

22. A method according to Claim 21 wherein said strain is a *Pseudomonas putida*.

23. A method according to Claim 21 wherein said strain is *Pseudomonas putida* IMI382568.

24. A method according to Claim 21, 22 or 23 wherein said strain is capable of producing both vanillic acid and vanillin from ferulic acid, the ratio thereof being pH-dependent; and wherein a pH is selected and maintained which relatively favours accumulation of vanillin.

25. A method according to any preceding claim wherein said second composition contains at least one desired component which is susceptible to further transformation; and wherein said transformation of said first composition is effected in an aqueous phase which is contacted with an organic phase which extracts said at least one desired component.

26. A method according to any preceding claim wherein said first composition contains ferulic acid and at least one further compound of the formula (A) where Z is neither CO_2H nor CO_2 *carb*, and wherein said at least one further compound also undergoes transformation.

27. A mixture resulting from the method of Claim 26 which includes vanillin.

28. A method according to any of claims 1 to 26 including a preliminary step of liberating said first composition from plant material.

29. A method according to any of Claims 1 to 26 including a preliminary step of obtaining said first composition comprising ferulic acid from a plant material by a process comprising:

(a) treating the plant material to produce a solution containing a ferulic acid ester; and

(b) treating said solution with an enzyme composition having ferulic acid esterase activity under conditions such that ferulic acid esters are converted into ferulic acid.

30. A method according to claim 28 or 29 wherein said plant material is selected from maize, wheat, sugar beet and rice materials.

31. A method according to claim 30 wherein said plant material comprises fibre, bran or straw.

32. A method according to Claim 29, 30 or 31 wherein in step (a) the plant material is treated with a solution containing citric acid or sodium bicarbonate.

33. A method according to claim 32 wherein said plant material is treated in the temperature range 50-250°.

34. A method according to Claim 29 wherein the plant material comprises sugar beet fibre and step (a) involves heating in water.

35. A method according to any of Claims 29 to 34 wherein step (b) employs an enzyme derived from a species of *Aspergillus* or *Humicola insolens*.

36. A method according to Claim 35 wherein the enzyme derived from *Humicola insolens* and treatment is effected at substantially in the pH range 6-7.

37. A method of obtaining a strain of microorganism for use in the method of any of Claims 1 to 26 or 28 to 36 comprising screening a multiplicity of colonies by means of a reagent suitable for detecting aldehydes.

38. A method according to Claim 37 wherein the multiplicity of colonies are obtained by mutation.

39. A method according to any of Claims 1 to 26 or 28 to 36 including a preliminary step of obtaining a microorganism for use therein by the method of Claim 37 or Claim 38.

40. *Pseudomonas putida* NCIMB40988 and mutants thereof capable of converting ferulic acid into vanillic acid.

41. *Rhodotorula glutinis* IMI 379896 and mutants thereof capable of converting ferulic acid into vanillic acid.

42. *Penicillium chrysogenum* IMI379900 and mutants thereof capable of converting a ferulic acid ester into vanillic acid.

43. *Aspergillus flavus* IMI379895 and mutants thereof capable of converting a ferulic acid ester into vanillic acid.

44. *Aspergillus niger* IMI379904 and mutants thereof capable of converting a ferulic acid ester into vanillic acid.

45. *Zygorhynchus moelleri* IMI379899 and mutants thereof capable of converting vanillic acid into vanillyl alcohol and vanillin.

46. *Pseudomonas putida* IMI382568 and mutants thereof capable of converting ferulic acid into vanillin.

47. *Aspergillus fumigatus* IMI379902 and mutants thereof capable of converting vanillic acid into vanillin.

48. *Micromucor isabellinus* IMI379893 and mutants thereof capable of converting vanillic acid into vanillin.

49. *Brevundimonas vesicularis* NCIMB40987 and mutants thereof capable of converting vanillyl alcohol into vanillin.

50. *Trichoderma koningii* IMI 379903 and mutants thereof capable of converting 4-hydroxylbenzoic acid into 4-hydroxybenzaldehyde.

51. An extract or enzyme(s) of an organism defined in any of claims 40-50 possessing the activity specified for the organism.

52. Microbial conversion of coumaric acid into p-hydroxybenzoic acid using *Rhodotorula glutinis* or *Pseudomonas putida* or an extract or enzyme(s) therefrom.

53. Microbial conversion of p-hydroxybenzoic acid into p-hydroxybenzyl alcohol using *Zygorhynchus moelleri* or an extract or enzyme(s) therefrom.

54. Microbial conversion of p-hydroxybenzoic acid into p-hydroxybenzaldehyde using *Trichoderma koningii* or *Zygorhynchus moelleri* or an extract or enzyme(s) therefrom.

55. Microbial conversion of p-hydroxybenzyl alcohol into p-hydroxybenzaldehyde using *Brevundimonas vesicularis* or an extract or enzyme(s) therefrom.

56. Microbial conversion of 3,4-dihydroxybenzoic acid into 3,4-dihydroxy benzaldehyde using *Zygorhynchus moelleri* or an extract or enzyme(s) therefrom.

57. Microbial conversion of benzoic acid into benzaldehyde using *Trichoderma koningii* or an extract or enzyme(s) therefrom.

58. Microbial conversion of ferulic acid into vanillic acid using *Pseudomonas putida* or *Rhodotorula glutinis* or an extract or enzyme(s) therefrom.

59. Microbial conversion of a ferulic acid ester into vanillic acid using *Penicillium chrysogenum*, *Aspergillus niger* or *Aspergillus flavus* or an extract or enzyme(s) therefrom.

60. Microbial conversion of vanillic acid into vanillin using *Aspergillus fumigatus* or *Micromucor isabellinus* or an extract or enzyme(s) therefrom.

61. Microbial conversion of ferulic acid into vanillin using *Pseudomonas putida* or an extract or enzyme(s) therefrom.

62. Microbial conversion of vanillic acid into vanillyl alcohol using *Zygorhynchus moelleri* or an extract or enzyme(s) therefrom.

63. Microbial conversion of vanillyl alcohol into vanillin using *Brevundimonas vesicularis* or an extract or enzyme(s) therefrom.

INTERNATIO[illegible]L SEARCH REPORT

Inter[illegible]nal Application No
PCT/GB 00/00654

A. CLASSIFICATION OF SUBJECT MATTER

IPC 7 C12P7/22 C12P7/24 C12P7/42 C12P39/00 C07C47/58 C12N15/01 C12N1/14 C12N1/16 //(C12P7/22,C12R1:40, C12R1:645,C12R1:68),(C12P7/24,C12R1:40,C12R1:645,C12R1:68),

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 7 C12P C07C C12N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X A	GB 2 301 103 A (DANISCO A/S) 27 November 1996 (1996-11-27) page 4 -page 10; claim 42	1-39, 51-63 40-50
X A	FAULDS C B ET AL: "RELEASE OF FERULIC ACID FROM WHEAT BRAN BY A FERULIC ACID ESTERASE (FAE-III) FROM ASPERGILLUS NIGER" APPLIED MICROBIOLOGY AND BIOTECHNOLOGY,DE,SPRINGER VERLAG, BERLIN, vol. 43, no. 6, 1 January 1995 (1995-01-01), pages 1082-1087, XP000603403 ISSN: 0175-7598 the whole document	1-39, 51-63 40-50
X	EP 0 885 968 A (GIVAUDAN-ROURE (INT.) S.A.) 23 December 1998 (1998-12-23) the whole document	1-39

-/--

☒ Further documents are listed in the continuation of box C.

☒ Patent family members are listed in annex.

° Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search 6 June 2000	Date of mailing of the international search report 13/06/2000
Name and mailing address of the ISA European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Authorized officer Douschan, K

INTERNATIONA[illegible]EARCH REPORT

Intern[illegible]nal Application No
PCT/GB 00/00654

A. CLASSIFICATION OF SUBJECT MATTER
IPC 7 (C12P7/42,C12R1:40,C12R1:645,C12R1:68)

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 94 13614 A (QUEST INT. B.V.) 23 June 1994 (1994-06-23) the whole document ---	1-39
X	WO 94 02621 A (V. MANE FILS S.A.) 3 February 1994 (1994-02-03) the whole document ---	1-39
X	WO 96 08576 A (I.N.R.A.) 21 March 1996 (1996-03-21) the whole document ---	1-39
X	EP 0 857 789 A (KYOWA HAKKO KOGYO CO., LTD.) 12 August 1998 (1998-08-12) claims 5-14 --- -/--	1-36

☒ Further documents are listed in the continuation of box C.

☒ Patent family members are listed in annex.

° Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search
6 June 2000

Date of mailing of the international search report

Name and mailing address of the ISA
European Patent Office, P.B. 5818 Patentlaan 2
NL – 2280 HV Rijswijk
Tel. (+31-70) 340-2040, Tx. 31 651 epo nl,
Fax: (+31-70) 340-3016

Authorized officer
Douschan, K

INTERNATIONAL SEARCH REPORT

International Application No
PCT/GB 00/00654

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 96 39859 A (ZYLEPSIS LTD.) 19 December 1996 (1996-12-19) cited in the application example 10 -----	37-39

INTERNATI[illegible]L SEARCH REPORT

Information on patent family members

Inter[illegible]nal Application No
PCT/GB 00/00654

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
GB 2301103 A	27-11-1996	NONE	
EP 0885968 A	23-12-1998	AU 7196498 A	24-12-1998
		BR 9802011 A	08-02-2000
		CA 2238215 A	19-12-1998
		CZ 9801883 A	13-01-1999
		JP 11069990 A	16-03-1999
		PL 326882 A	21-12-1998
		ZA 9805146 A	21-12-1998
WO 9413614 A	23-06-1994	AU 5698794 A	04-07-1994
WO 9402621 A	03-02-1994	FR 2694020 A	28-01-1994
		AT 173296 T	15-11-1998
		DE 69322067 D	17-12-1998
		DE 69322067 T	15-07-1999
		EP 0606441 A	20-07-1994
		JP 7500253 T	12-01-1995
		US 5712132 A	27-01-1998
WO 9608576 A	21-03-1996	FR 2724394 A	15-03-1996
		AT 174632 T	15-01-1999
		DE 69506724 D	28-01-1999
		DE 69506724 T	01-07-1999
		EP 0781345 A	02-07-1997
		ES 2128074 T	01-05-1999
		US 5866380 A	02-02-1999
EP 0857789 A	12-08-1998	CA 2228816 A	07-08-1998
		CN 1206045 A	27-01-1999
		JP 10276788 A	20-10-1998
		US 5955137 A	21-09-1999
WO 9639859 A	19-12-1996	AU 2626895 A	04-01-1996
		AU 6008996 A	30-12-1996
		EP 0764147 A	26-03-1997
		EP 0831715 A	01-04-1998
		JP 11506339 T	08-06-1999
		JP 10501216 T	03-02-1998

FOR THE PURPOSES OF INFORMATION ONLY

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AL	Albania
AM	Armenia
AT	Austria
AU	Australia
AZ	Azerbaijan
BA	Bosnia and Herzegovina
BB	Barbados
BE	Belgium
BF	Burkina Faso
BG	Bulgaria
BJ	Benin
BR	Brazil
BY	Belarus
CA	Canada
CF	Central African Republic
CG	Congo
CH	Switzerland
CI	Côte d'Ivoire
CM	Cameroon
CN	China
CU	Cuba
CZ	Czech Republic
DE	Germany
DK	Denmark
EE	Estonia
ES	Spain
FI	Finland
FR	France
GA	Gabon
GB	United Kingdom
GE	Georgia
GH	Ghana
GN	Guinea
GR	Greece
HU	Hungary
IE	Ireland
IL	Israel
IS	Iceland
IT	Italy
JP	Japan
KE	Kenya
KG	Kyrgyzstan
KP	Democratic People's Republic of Korea
KR	Republic of Korea
KZ	Kazakstan
LC	Saint Lucia
LI	Liechtenstein
LK	Sri Lanka
LR	Liberia
LS	Lesotho
LT	Lithuania
LU	Luxembourg
LV	Latvia
MC	Monaco
MD	Republic of Moldova
MG	Madagascar
MK	The former Yugoslav Republic of Macedonia
ML	Mali
MN	Mongolia
MR	Mauritania
MW	Malawi
MX	Mexico
NE	Niger
NL	Netherlands
NO	Norway
NZ	New Zealand
PL	Poland
PT	Portugal
RO	Romania
RU	Russian Federation
SD	Sudan
SE	Sweden
SG	Singapore
SI	Slovenia
SK	Slovakia
SN	Senegal
SZ	Swaziland
TD	Chad
TG	Togo
TJ	Tajikistan
TM	Turkmenistan
TR	Turkey
TT	Trinidad and Tobago
UA	Ukraine
UG	Uganda
US	United States of America
UZ	Uzbekistan
VN	Viet Nam
YU	Yugoslavia
ZW	Zimbabwe

PCT

WORLD INTELLECTUAL PROPERTY ORGANIZATION
International Bureau

INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 7 :
C12P 7/22, 7/24, 7/42, 39/00, C07C 47/58, C12N 15/01, 1/14, 1/16 // (C12P 7/22, C12R 1:40, 1:645, 1:68) (C12P 7/24, C12R 1:40, 1:645, 1:68) (C12P 7/42, C12R 1:40, 1:645, 1:68)

A1

(11) International Publication Number: **WO 00/50622**

(43) International Publication Date: 31 August 2000 (31.08.00)

(21) International Application Number: PCT/GB00/00654

(22) International Filing Date: 24 February 2000 (24.02.00)

(30) Priority Data:
9904251.7 24 February 1999 (24.02.99) GB

(71) Applicant *(for all designated States except US)*: ZYLEPSIS LIMITED [GB/GB]; 6 Highpoint, Henwood Business Estate, Ashford, Kent TN24 8DH (GB).

(72) Inventors; and
(75) Inventors/Applicants *(for US only)*: CHEETHAM, Peter, Samuel, James [GB/GB]; 3 Wallace Close, Tunbridge Wells, Kent TN2 5HW (GB). GRADLEY, Michelle, Lorraine [GB/GB]; 22A Nunnery Road, Canterbury, Kent CT1 3LU (GB). SIME, John, Thomas [GB/GB]; "Lallans", 31A Canon Woods Way, Kennington, Ashford, Kent TN24 9QY (GB).

(74) Agents: STUART, Ian et al.; Mewburn Ellis, York House, 23 Kingsway, London WC2B 6HP (GB).

(81) Designated States: AE, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CR, CU, CZ, DE, DK, DM, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, TZ, UA, UG, US, UZ, VN, YU, ZA, ZW, ARIPO patent (GH, GM, KE, LS, MW, SD, SL, SZ, TZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG).

Published
With international search report.

(54) Title: FLAVOUR/AROMA MATERIALS AND THEIR PREPARATION

(57) Abstract

Flavour components, particularly for a vanilla composition, are produced by essentially natural methods employing biotransformation of plant-derived materials. Ferulic acid, a component of many plant cell walls, may be converted into vanillin, directly or indirectly. A plurality of such compounds may undergo bioconversions to produce components of a flavour composition.

FOR THE PURPOSES OF INFORMATION ONLY

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AL	Albania
AM	Armenia
AT	Austria
AU	Australia
AZ	Azerbaijan
BA	Bosnia and Herzegovina
BB	Barbados
BE	Belgium
BF	Burkina Faso
BG	Bulgaria
BJ	Benin
BR	Brazil
BY	Belarus
CA	Canada
CF	Central African Republic
CG	Congo
CH	Switzerland
CI	Côte d'Ivoire
CM	Cameroon
CN	China
CU	Cuba
CZ	Czech Republic
DE	Germany
DK	Denmark
EE	Estonia
ES	Spain
FI	Finland
FR	France
GA	Gabon
GB	United Kingdom
GE	Georgia
GH	Ghana
GN	Guinea
GR	Greece
HU	Hungary
IE	Ireland
IL	Israel
IS	Iceland
IT	Italy
JP	Japan
KE	Kenya
KG	Kyrgyzstan
KP	Democratic People's Republic of Korea
KR	Republic of Korea
KZ	Kazakstan
LC	Saint Lucia
LI	Liechtenstein
LK	Sri Lanka
LR	Liberia
LS	Lesotho
LT	Lithuania
LU	Luxembourg
LV	Latvia
MC	Monaco
MD	Republic of Moldova
MG	Madagascar
MK	The former Yugoslav Republic of Macedonia
ML	Mali
MN	Mongolia
MR	Mauritania
MW	Malawi
MX	Mexico
NE	Niger
NL	Netherlands
NO	Norway
NZ	New Zealand
PL	Poland
PT	Portugal
RO	Romania
RU	Russian Federation
SD	Sudan
SE	Sweden
SG	Singapore
SI	Slovenia
SK	Slovakia
SN	Senegal
SZ	Swaziland
TD	Chad
TG	Togo
TJ	Tajikistan
TM	Turkmenistan
TR	Turkey
TT	Trinidad and Tobago
UA	Ukraine
UG	Uganda
US	United States of America
UZ	Uzbekistan
VN	Viet Nam
YU	Yugoslavia
ZW	Zimbabwe

FLAVOUR/AROMA MATERIALS AND THEIR PREPARATION

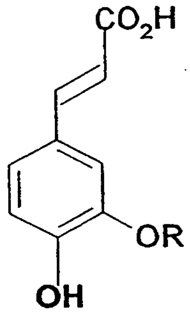
Background

The present invention relates to flavour/aroma materials and the preparation of such materials and key intermediates. It is particularly (though not exclusively) concerned with vanilla flavour materials and related materials.

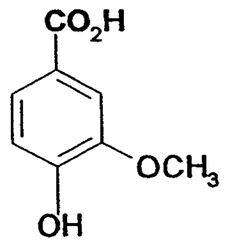
Supplies of natural vanilla bean extracts suffer from shortages of supply and variability in quality. Despite a wide range of natural flavour chemicals having become commercially available over the last 10-15 years, no entirely satisfactory natural vanillin flavour chemical product or vanilla flavour has yet been developed. The main difficulties in developing a cost-effective vanillin product are firstly the unavailability of the preferred raw material, ferulic acid; and secondly the difficulty in finding microbial strains that can accumulate vanillin due to its ease of further metabolism, for instance to vanillic acid, and its inhibitory effect on the metabolism of cells. The main difficulty in developing a natural vanilla flavour is the large number of different chemicals that together contribute to the superior flavour and aroma of vanilla bean extracts. In addition for the minor usage of vanilla in fragrances a colourless solid product is required rather than the coloured ethanol-water vanilla bean extracts.

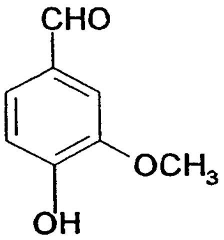
Our earlier application WO-A-96/39859 discloses the production of some phenolic materials by the enzymatic hydrolysis of plant materials. Thus ferulic acid (1a) was produced by enzyme treatment of wheat germ or wheat bran.

Caffeic acid (1b) was produced by enzyme treatment of sunflower meal. Ferulic acid and esters thereof are valuable as precursor compounds and also as ingredients of foods and cosmetics, e.g. serving as antioxidants.


(1a)R-CH$_3$

(1b)R=H

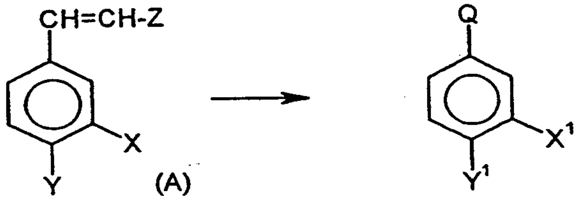

(2)


(3)

Various workers have reported the microbial conversion of ferulic acid (1a) into vanillin (3) either directly (e.g. DE-A-19532317) or via vanillic acid (2) (FR-A-2724394).

Outline of the Invention

The invention provides, inter alia, a method of converting a first composition comprising one or more species of formula (A) into a second composition comprising one or more species of formula (B):


(A) (B)

where X, Y, X' and Y' are independently selected from H, OH and OMe; Z is CO_2H, CO_2*carb* (where *carb* represents a carbohydrate residue), CHO or CH_2OH, and Q is CHO, CO_2H or CH_2OH, said method comprising treating said first composition with one or more microorganisms under conditions such that

(A) is converted into (B); said microorganism(s) being selected, from (a) *Pseudomonas putida*; (b) *Rhodotorula* species and other yeasts capable of converted ferulic acid into vanillic acid; (c) microorganisms possessing both ferulic acid esterase activity and intra-sidechain cleavage activity such that they are capable of converting ferulic acid glycosides into vanillin and/or vanillic acid; and (d) *Micromucor isabellinus* or *Aspergillus fumigatus* strains capable of converting vanillic acid into vanillin.

The process may include a preliminary step of obtaining said first composition comprising ferulic acid from a plant material by a process comprising:

(a) treating the plant material to produce a solution containing a ferulic acid ester; and

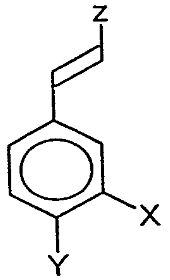
(b) treating said solution with an enzyme composition having ferulic acid esterase activity under conditions such that ferulic acid esters are converted into ferulic acid. N.B the esters generally involve carbohydrate residues, particularly sugar residues, and are sometimes termed glycosides.

The invention further provides several microorganisms useful in methods of the invention. These include several deposited strains and mutants thereof (which may be produced by chemical or other conventional mutagenesis or by genetic engineering). As well as intact organisms, use may be made of extracts and isolated enzymes.

Aspects of the present invention include the following.

A) A process for use in preparing a flavour/aroma composition containing a plurality of flavour/aroma components, said process having the step(s) of

(i) treating a plant material or plurality of plant materials to produce a precursor compound and preferably a plurality of precursor compounds (separately or mixed) preferably comprising two or more 1-phenylalkene species, preferably of formula (4):



(4)

where X and Y are independently selected from H, OH and OMe and Z is CO_2H, CO_2*carb* (where *carb* represents a carbohydrate residue) CHO or CH_2OH, most preferably comprising the compound (4) wherein X=OMe, Y=OH and Z=CO_2H (ferulic acid) and preferably also the compounds wherein X=H, Y=OH and Z=CO_2H (coumaric acid) and X=OH, Y=OH and Z=CO_2H (caffeic acid) ; and preferably

ii) subjecting said precursor compound or compounds (with or without separation from the plant material residues) to one or more biotransformations to produce a flavour/aroma composition. For precursor compounds having a benzene ring bearing a substituent -CH:CH-CO_2H (e.g. compounds of structure (4)), biotransformations may generate compounds in which this substituent has been converted into -CO_2H and/or -CH_2OH and/or -CHO.

The term "plant material" as used herein includes material such as the meal or pulp produced by mechanical processing of plants, and the residues left after extraction of seed oils etc..

Particularly preferred plant materials for step (i) include maize, wheat, rice, sugar beet and parts thereof, particularly waste materials from their normal uses e.g. rice bran and cereal fibres. For example maize fibre and wheat fibre may be derived from dry or wet milling. Sugar beet fibre may be derived from pulp. A mixture of plant materials may be employed to give a desired mixture of precursors.

Step (i) may involve:

(a) treatment of plant material (damp or dried) with acid (preferably citric acid, e.g. provided by addition of lemon or lime juice) to release glycosides of at least ferulic acid, generally with heating e.g. to 50-250°; and

(b) treatment of the glycoside-containing mixture with base (e.g. an alkali metal bicarbonate) or one or more enzymes with ferulic acid esterase ("FAE") activity to release ferulic acid. Suitable enzymes include Hemicellulase (from Amano, derived from <u>Aspergillus</u> spp.) and/or Celluzyme (Novo Nordisk) and/or enzymes from <u>Humicola insolens</u>, available as Pentopan, Biofeed Plus or Biofeed Beta (Novo Nordisk). These enzymes have some xylanase activity, in addition to their FAE activity. If required, additional sources of xylanase can be added to supplement the xylanase activity already present. (Note: the so-called glycosides of ferulic acid etc. are in fact esters of carbohydrate residues rather than conventional glycoside ethers).

In step (a), citric acid is suitable because it is reasonably strong, heat stable, cheap, active on a range of ferulic acid containing materials, non-volatile, and not inclined to cause side reactions. It is adequately soluble in the cereal "mashes". It can easily be recovered as an insoluble salt (e.g. calcium), for reuse. It is a "natural" material, which is approved for food use. Alternatives include other organic polycarboxylic acids, particularly hydroxyacids, such as isocitric, tartaric, malic, fumaric and succinic acids.

Solutions containing suitable acids may be used, e.g. a grape-derived solution containing tartaric acid; or a fermentation medium containing citric or malic acid.

A microorganism may provide the activities required to carry out step (i)(b) and step (ii) (biotransformation of ferulic acid and/or other precursor compounds). For example we have developed strains of Aspergillus niger, A.flavus, and Penicillium chrysogenum having both the necessary FAE and alkene cleavage activities for acting on the product of the step (a) to convert ferulic acid glycoside into vanillic acid.

Step (i) can also be effected by treatment of plant material (e.g. maize fibre) with aqueous alkali such as a hydroxide, carbonate or bicarbonate of an alkali metal or alkaline earth metal. Sodium bicarbonate is preferred.

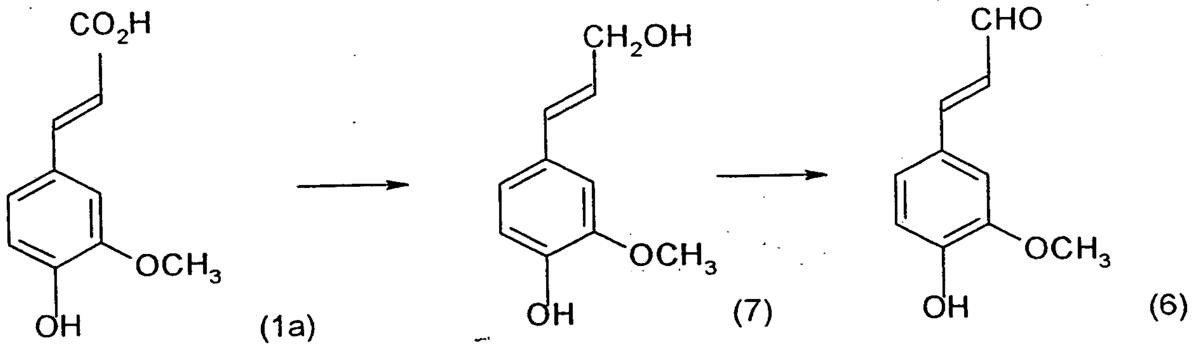
A product mixture containing vanillic acid and other materials (e.g. p-hydroxybenzoic acid from coumaric acid) may undergo one or more further biotransformations without isolation of individual components, e.g. converting vanillic acid into vanillin and effecting corresponding transformations of other components.

B) Preparation of vanillin comprising the biotransformation of vanillic acid (2) into vanillyl alcohol (5), and the biotransformation of vanillyl alcohol (5) into vanillin (3). Generally the two steps will employ different microorganisms. N.B compounds such as vanillyl alcohol may be valuable flavour chemicals in their own right.

The steps may be novel in their own right. The reduction of vanillic acid to vanillyl alcohol may be effected by Zygorhynchus moelleri.

C) Preparation of vanillin comprising the biotransformation of vanillic acid (2) directly into vanillin (3) by means of strains of microorganisms such as Aspergillus fumigatus or Micromucor isabellinus.

D) Preparation of vanillin comprising the biotransformation of ferulic acid into vanillin, e.g. by a strain of Pseudomonas putida.



E) Preparation and isolation of substances, particularly substances prone to further reaction (e.g. aldehydes) or prone to inhibiting microorganisms (e.g. vanillin) by effecting a biotransformation using a microorganism in an aqueous phase which is brought into contact with a second phase into which products pass. This "<u>in situ</u> product removal" ("ISPR") may employ a vegetable oil as the second phase. Products may subsequently be recovered from the second phase, e.g. by crystallisation or solvent extraction. This process of ISPR can be employed in step (ii) of process A (above), or in processes B, C and D.

F) A vanilla flavour/aroma composition which is the product of a process according to (A) above or is a blend of one or more such products and/or one or more substances prepared by a process according to (B), (D), or (E), above and/or one or more vanilla flavour chemicals from other sources and/or a vanilla bean extract.

G) Use of <u>Pseudomonas putida</u> for converting ferulic acid into vanillic acid. The raw material is preferably the mixture obtained by treating plant material such as cereal fibre with citrate and FAE enzyme (as described in section A above), without isolation of the ferulic acid.

H) Various transformations as described above may be applied to other substrates. This may lead to further useful flavour/odour components. For example benzoic acid, 4-hydroxybenzoic acid and 3,4-dihydroxybenzoic acids can be converted into the corresponding aldehydes and/or benzyl alcohols.

I) A method of isolating strains of microorganisms for use in processes of the invention. Material (e.g. soil samples) containing a multiplicity of strains is used to produce a multiplicity of colonies, e.g. on agar, and individual colonies are tested for useful activity by means of a reagent suitable for detecting aldehydes. For example, 2,4-dinitro-phenylhydrazine produces orange/red zones around aldehyde-producing colonies and a dark yellow zone around an alcohol producing colony.

J) Strains isolated by method I) and mutants thereof (obtained naturally, by conventional mutagenesis or by genetic engineering). This includes heterologous organisms which have been transformed so that they have derived activities of the "parent" organism from which the transforming nucleic acid was directly or indirectly derived.

Some particularly preferred strains have been deposited under the Budapest Treaty. Brief details follow.

a) Strains deposited with NCIMB (NCIMB Ltd, 23 St Machar Drive, Aberdeen, A24 3RY, GB)

1. Brevundimonas vesicularis (Zyl 295)-NCIMB 40987-Gram negative bacterium (deposited 18/11/98)

2. Pseudomonas putida (Zyl 503)-NCIMB 40988-Gram negative bacterium (18/11/98)

b) Strains deposited with IMI (CABI Bioscience UK Centre Egham, Genetic Resource Collection, Bakeham Lane, Egham, Surrey TW20 9TY, GB)

1. Rhodotorula glutinis (Zyl 717)-IMI 379896-Yeast, producing a red/orange pigment (20/11/98)

2. Aspergillus flavus (Zyl 714)-IMI 379895-Filamentous fungus producing light green spores (20/11/98)

3. Aspergillus fumigatus (Zyl 747)-IMI 379902-Filamentous fungus producing blue/grey spores (20/11/98)

4. Trichoderma koningii (Zyl 751)-IMA 379903-Filamentous fungus producing green spores (20/11/98)

5. Aspergillus niger (Zyl 759)-IMI 379904-Filamentous fungus producing brown/black spores (20/11/98)

6. Micromucor isabellinus (Zyl 849)-IMI 379893-Filamentous fungus producing pale brown spores (20/11/98)

7. Zygorhynchus moelleri (Zyl 851)-IMI 379899-Filamentous fungus, with aerial hyphae producing black spores (20/11/98)

8. Penicillium chrysogenum (Zyl 860)-IMI 379900-Filamentous fungus producing blue spores (20/11/98)

9. Pseudomonas putida (Zyl 581)-IMI 382568, Gram negative bacterium; deposited 31/1/00

The use of those strains is illustrated in the following examples. Of course other strains of the same species may be used to carry out the same transformations.

The invention will now be explained in more detail, with reference to some specific examples.

General Experimental Conditions

In the following examples, where organisms are grown in culture broth, the growth medium can contain specified amounts of either, or both, of a vitamins supplement and a trace elements supplement.

These were prepared as follows.

Vitamins supplement: biotin (2mg L^{-1}), folic acid (2mg L^{-1}), pyridoxine (10mg L^{-1}), riboflavin (5mg L^{-1}), thiamine (5mg L^{-1}), nicotinic acid (5mg L^{-1}), pantothenic acid (5mg L^{-1}), vitamin B12 (0.1mg L^{-1}), 4-aminobenzoic acid (5mg L^{-1}), and thioacetic acid (5mg L^{-1}).

Trace elements supplement: concentrated hydrochloric acid (51.3 mL L^{-1}), MgO (10.75g L^{-1}), $CaCO_3$ (2.0g L^{-1}), $FeSO_4.7H_2O$ (4.5g L^{-1}), $ZnSO_4.7H_2O$ (1.44 g L^{-1}), $MnSO_4.4H_2O$ (1.12 g L^{-1}), $CuSO_45H_2O$ (0.25g L^{-1}), $CoSO_4.7H_2O$ (0.28g L^{-1}), and H_3BO_3 (0.06 g L^{-1}).

Analysis of coniferyl alcohol, coniferaldehyde, caffeic acid, coumaric acid, ferulic acid, vanillic acid, vanillyl alcohol and vanillin was carried out using high performance liquid chromatography (hplc) using the following conditions:

Column	Spherisorb C_{18}
Mobile phase	80:20 deionised water:acetonitrile containing 1% acetic acid

Flow rate	1.75 mL min^{-1}
Detection	Ultraviolet at 290nm

Analysis of 4-hydroxybenzoic acid and 4-hydroxybenzaldehyde was carried out using high performance liquid chromatography (hplc) using the following conditions:

Column	Spherisorb C_{-18}
Mobile phase	80:20 deionised water:acetonitrile containing 1% acetic acid
Flow rate	2 mL min^{-1}
Detection	Ultraviolet at 275nm

Alternatively, analysis of 4-hydroxybenzoic acid and 4-hydroxybenzaldehyde was carried out using thin layer chromatography (tlc) using the following conditions: silica plates eluting with petroleum either (40-60): ethyl acetate (50:50) and visualisation with UV or dinitrophenylhydrazine solution (0.4% in 2M HC1).

A) <u>Use of Plant Materials</u>

Maize fibre is an example of a cheap raw material which can provide raw materials for use in the preparation of flavour/aroma materials. Treatment with alkali leads to the liberation of ferulic acid, and lesser amounts of other materials, notably coumaric acid. The mixture can be subjected to biotransformation, e.g. to produce a mixture of vanillic acid and 4-hydroxybenzoic acid, which could be subjected to further biotransformations, e.g. converting the carboxylic acid groups to -CHO and/or $-CH_2OH$.

<u>Examples 1A: Ferulic and coumaric acids from maize</u>

(a) Use of sodium hydroxide: to 500g of ground maize fibre was added 1 litre of 1M sodium hydroxide solution and the resulting suspension was thoroughly mixed then allowed to stand at ambient temperature (22°C) for 15 hours. Then 1 litre of ethyl acetate and 100mL of concentrated hydrochloric acid were added and the suspension mixed. The ethyl acetate phase was separated and the fibre suspension re-extracted with a further 1 litre of ethyl acetate. The combined organic solvent phases were dried (Na_2SO_4) and evaporated to dryness to yield a thick oil. Repeated washing of this oil with n-hexane gave a pale yellow solid comprising 33% ferulic acid and 2.9% coumaric acid.

(b) Use of sodium bicarbonate: to 10g maize fibre containing 1.75%w/w ferulic acid (FA) and 0.1%w/w coumaric acid (CA) was added 100ml 0.5m sodium bicarbonate solution in a 250ml conical flask. The resulting suspension was heated and mixed at 85°C using a hot plate stirrer apparatus. The release of ferulic acid and coumaric acid into solution was monitored over time using hplc. Ferulic acid and coumaric acid yields were as follows:

60 min., FA 20mg, CA 1.6mg; 245 min., FA 100mg, CA 4.3mg; 315 min, FA 127mg, CA 5.6mg; 365 min., FA 126mg, CA 6.2mg. Yields at 365 min are equivalent to 72% and 62% release of available ferulic acid and coumaric acid respectively.

The maize suspension was coarse filtered by pressing through a mesh bag, the recovered solids washed with 10ml deionised water and the filtrates combined prior to centrifugation (4,000 x g, 15 min). The pH of the supernatant was adjusted to pH 2.5 with concentrated hydrochloric acid followed by extraction with ethyl acetate (3x100ml). Evaporation of the combined ethyl acetate layers to dryness yielded 216mg of yellow/orange solid material comprising 41% ferulic acid and 2.7% coumaric acid.

In the following examples (c) and (d), the production of ferulic acid and coumaric acid from maize fibre was effected by a two step process. Firstly, acid hydrolysis of the fibre was achieved by heating with citric acid solutions, supplied as either a defined quantity of citric acid dissolved in water or as the juice from freshly squeezed lemon or lime fruit. Secondly, the hydrolysis of solubilised cinnamate sugar esters was achieved by the addition of a hydrolytic enzyme preparation yielding ferulic and coumaric acids.

(c) Maize fibre (20g) was mixed with 100ml of citric acid solution (2%) in 250ml conical flask and heated at 126°C for 1 hour. The pH of the maize suspension was raised to pH 5.0 by the addition of 10M sodium hydroxide solution with vigorous mixing. An enzyme preparation (40mg, Hemicellulase, Amano) was added to the suspension and the whole incubated at 50°C for 46.5 hours with mixing at 200rpm. The release of ferulic acid was monitored by hplc as described above. After 1.5 hours incubation a total of 51mg ferulic acid was present in solution, after 46.5 hours this amount had risen to 220 mg.

(d) Maize fibre (200g) was mixed with 1 litre citric acid solution (2%) in a 2L conical flask and heated at 126°C for 1 hour. The maize suspension was separated into insoluble solids and a liquor fraction using a wine press. First pressing yielded approximately 800ml liquor; the recovered solids were washed with a further 200ml of water and pressed again to give approximately 1 litre of combined liquor fractions. The pH of the maize liquor was raised to pH 7.0 by the addition of 10M sodium hydroxide with vigorous mixing. An enzyme preparation (Biofeed Plus L,2ml,Novo Nordisk) was added to the liquor and the whole incubated at 60°C for 7.5 hours with mixing at 160 rpm. The release of ferulic acid and coumaric acid was monitored by hplc as described above. After 7.5 hour incubation 1.6g L^{-1} ferulic acid and 0.1 gL^{-1} p-coumaric acid were detected in solution. Both cinnamic acids were recovered from aqueous solution by extraction into ethyl acetate as described below, followed by either, base extraction of the solvent to yield the cinnamate sodium salt, or evaporation of the solvent to dryness to yield the cinnamate free acid.

Maize liquor (1L) was brought to pH 3 by the addition of concentrated hydrochloric acid and filtered through diatomaceous earth to remove insoluble material. The filtrate was extracted twice with 300 ml of ethyl acetate and the solvent extracts combined. Evaporation of solvent to dryness yielded 2.6g of orange solid comprising 1.39g ferulic acid and 0.1g coumaric acid, 53.5% and 3.8% purity respectively. In order to recover the cinnamates as their sodium salts, the ethyl acetate was continuously pumped over 10M sodium hydroxide solution (10ml) combined with vigorous mixing of the aqueous phase. This was continued until all but trace quantities of cinnamate had been recovered into the aqueous phase (approx. 2hrs). The aqueous phase was dried under vacuum at 45°C to yield 11.5 g of cream solid material comprising 1.3g ferulic acid and 0.1g coumaric acid, 11.3% and 0.87% purity respectively.

e) <u>Conversion of citric acid treated maize fibre suspension to a digested pulp containing ferulic acid and vanillic acid</u>

4g maize fibre containing ca 1.75% w/w ferulic acid and 20 ml 2% w/v citric acid solution were added to each of three 50ml conical flasks and the whole autoclaved at 126°C for 55 minutes. After cooling the pH of the treated suspension was adjusted to pH 6.0 using 10M sodium hydroxide solution. Flasks were inoculated with spores of either Zyl 714 (IMI 379895) *Aspergillus flavus*, Zyl 759 (IMI 379904) *Aspergillus niger* or *P.chrysogenum* Zyl 860 (IMI 379900) and incubated at 30°C, shaking at 250 rpm on an orbital mixer. Flasks were assayed by hplc for the hydrolytic product ferulic acid, and for the ferulic acid side chain cleavage product vanillin acid.

After 89 hours incubation the concentrations of ferulic acid and vanillic acid in solution for the *P.chrysogenum* experiment were ferulic acid, undetectable; and vanillic acid, 1.35g/L.

Concentrations of ferulic acid and vanillic acid in solution for *Aspergillus flavus*, *Aspergillus niger* respectively after 5 days incubation were 1g/L ferulic acid and 0.3g/l vanillic acid; undetectable ferulic acid and 1.2g/L vanillic acid.

All three fungi reduced the maize fibre suspension to a digested pulp and the fibre fragments to fine solids.

f) Pilot scale processing of maize fibre for release of ferulic acid

3 batches of 10kg maize fibre were each suspended in 50L of 2% w/v aqueous citric acid solution. Batch 1 and 2 were heated to 126° for 1 hour and then rapid cooled to 80°C using an Agemore swept surface heating/mixing vessel. The solids were then separated from the liquor using a Vigo 72L winepress, and the solids washed with water to restore the starting volume.

Batch 1 and 2 combined generated 100L of liquor. A sample of this liquor was treated by base hydrolysis to release the available ferulic acid and analysed by hplc , the ferulic acid concentration was $1.5gL^{-1}$.

The third batch was heated to 134°C for 15 minutes, rapid cooled to 80°C, and pressed using the same apparatus as batches 1 and 2, however the solids were not washed with water after the first pressing. This batch generated 44.5L of liquor. A sample of this liquor was treated by base hydrolysis to release the available ferulic acid and analysed by hplc, the ferulic acid concentration was $1.12gL^{-1}$.

All three batches of liquor were then combined to give 145L and the concentration of suspended solids measured as 10.5% w/v by refractometry, the pH was measured as pH 3.4. The liquor was then concentrated using a Junior rising/falling plate evaporator at 60°C. 19.5 L of liquor was obtained, the suspended solids level was determined, by refractometry, to be 50%. A sample of this liquor was treated by base hydrolysis to release the available ferulic acid and analysed by hplc, the ferulic acid concentration was 8.0gL^{-1}.

The concentrated liquor was then transferred to a 70L Biolafitte fermenter and diluted with water to give a total volume of 33L. The pH of the liquor was adjusted from pH 3.0 to pH 6.0 with 2.5L 32% w/v sodium hydroxide solution. The liquor was then heated to 45°C. An enzyme, hemicellulase (Amano), was added at a concentration of 0.8gL^{-1}, and the mixture incubated with stirring for 30 hours. After the enzyme treatment, the liquor was analysed by hplc, the ferulic acid concentration was 3.5gL^{-1}.

The pH of the liquor was lowered to pH 3.0 with 82.5% w/v phosphoric acid and the suspended solids removed by centrifugation using a Carr Powerfuge. The ferulic acid was then extracted from the liquor using 50L of butyl acetate (in two batches of 25L). The butyl acetate was overlaid on the liquor and mixed gently in the fermenter, until the concentration of ferulic acid in the butyl acetate layer stopped increasing. 48L of butyl acetate were recovered once the extraction was complete. Analysis of the butyl acetate by hplc gave the ferulic acid concentration as 2.64gL^{-1}.

g) <u>Pilot scale processing of sugar beet for release of ferulic acid</u>

40kg Fibrex sugar beet fibre was suspended in 200L water overnight. This suspension was then heated to 134°C for 30 minutes and then rapid cooled to 80°C (cooling time was 10 minutes) using an Agemore swept surface heating/mixing vessel. The solids were then separated from the liquor using a Vigo 72L winepress, and the solids washed with water to restore the starting volume.

200L of liquor was obtained, the pH was measured as pH 4.0, the suspended solids level was determined, by refractometry, to be 9.5% w/v. A sample of this liquor was treated by base hydrolysis to release the available ferulic acid and analysed by hplc, the ferulic acid concentration was $1.03gl^{-1}$.

The liquor obtained was then concentrated using a Junior rising/falling plate evaporator at 60°C. 31L of liquor was obtained, the suspended solids level was determined, by refractometry, to be 50% w/v. A sample of this liquor was treated by base hydrolysis to release the available ferulic and analysed by hplc, the ferulic acid concentration was $5.2gL^{-1}$.

The liquor was then transferred to a 70L Biolafitte fermenter and heated to 60°. The pH was adjusted to, and controlled at, pH 6.5 with 20% w/v phosphoric acid. An enzyme, Pentopan BG 500 (Novo Nordisk), was added at a concentration of $1.8gL^{-1}$, and the mixture incubated, with stirring, for 24 hours. After the enzyme treatment, the liquor was analysed by hplc, the ferulic acid concentration was $2.55gL^{-1}$.

The pH of the liquor was lowered to pH 3.0 with 82.5% w/v phosphoric acid and the suspended solids removed by centrifugation using a Carr Powerfuge. The ferulic acid was then extracted from the liquor using 35L of butyl acetate (in three batches, 2 x 15L, 1 x 5L). The butyl acetate was overlaid on the liquor and mixed gently in the fermenter, until the concentration of ferulic acid in the butyl acetate layer stopped increasing. 32L of butyl acetate were recovered once the extraction was complete. Analysis of the butyl acetate by hplc gave the ferulic acid concentration as $2.10gL^{-1}$.

The ferulic acid was then extracted from the butyl acetate by base extraction. The butyl acetate was pumped through 50ml of deionised water in a 500ml Duran bottle. The water was maintained at pH 9.0 using a Metrohm pH Stat titrating 10M sodium hydroxide and mixed using a magnetic stirrer plate and stirrer bar. Once it had passed through the aqueous phase, the butyl acetate was pumped back into the bulk solvent volume. This arrangement extracts the ferulic acid from the solvent phase and concentrates it in the pH 9.0 aqueous phase. The aqueous phase eventually became saturated with ferulic acid and a precipitate was formed which was recovered by filtration under vacuum through Whatman no.41 filter papers. These solids were dried overnight at 50°C in a drying oven. The first batch of solids obtained by this method weighed 34g and contained 24g ferulic acid by hplc analysis. The second batch of solids weighed 18g and contained 8g ferulic acid by hplc analysis.

h) <u>Pilot scale processing of maize fibre for release of ferulic acid by base hydrolysis</u>

1 tonne of maize fibre was suspended in 10,000L of water containing 600 Kg 30.5% sodium hydroxide. This was then heated to 50°C in a $15m^3$ stainless steel jacketed vessel and mixed for 8 hours. The pH of this suspension was measured as pH 11.8.

The bulk solids were then removed using a scroll decanter, and the liquor obtained transferred to a storage vessel. The pH of the liquor was then adjusted to pH 6.0 with 160 Kg 75% w/v phosphoric acid.

The liquor was then concentrated using an APV evaporator, which gave a final product of 2.5 tonnes. After concentration, the pH of the liquor was lowered to pH 3.0 with 180 Kg 75% w/v phosphoric acid. A sample of this material was removed and analysed for ferulic acid by hplc, the ferulic acid concentration was $2.1gKg^{-1}$.

i) <u>Repeat use of citric acid solutions for extraction of ferulic acid from maize fibre</u>

20g of Citric acid was dissolved in 1 litre of RO water, pH was measured as pH 2.20. 200g of maize fibre (supplied by Staley) was added to the citric acid solution and then autoclaved at 121°C for 60 minutes. The solids were then separated from the liquor using a Vigo bench wine press whilst the suspension was still at 80°C. 800ml of liquor was recovered from the initial pressing. The retained solids were then washed with 200 ml RO water, and pressed a second time. 220 ml of liquor was recovered from the second pressing. The liquor obtained from both pressings were added together to give a total volume of 1.02 L. The pH of the liquor was measured as pH 2.32.

A sample of liquor was treated by base hydrolysis to release the available ferulic acid and analysed by hplc, the ferulic acid concentration was $2.49gL^{-1}$. This represents a yield of 1.24% w/w ferulic acid from maize fibre.

The pH of the liquor was returned to pH 2.20 using 2.37g of citric acid. A fresh 200g of maize fibre (supplied by Staley) was added to the liquor and then autoclaved at 121°C for 60 minutes. The suspension was treated as described above, 730ml was recovered from the first pressing, the solids were washed with 270ml of RO water and pressed again, 320ml was recovered from the second pressing. The liquors obtained from both pressings were added together to give a total volume of 1.05L. The pH of the liquor was measured as pH 2.58.

A sample of liquor was treated by base hydrolysis to release the available ferulic acid and analysed by hplc, the ferulic acid concentration was $3.13gL^{-1}$. This represents a yield of 0.82% w/w ferulic acid from maize fibre.

Example 1B: biotransformation of product mixtures

A seed stage culture of Rhodotorula glutinis (Zyl 717) was grown for 24 hours at 30°C with shaking at 200 rpm in a 250 mL shake flask containing 50 mL of minimal medium (containing 2g/l KH_2PO_4; 0.2g/l NaCl; 0.22g/l $MgSO_4$; 0.015g/l $CaCl_2$; 1ml/l trace elements solution; 10ml/l vitamins solution; 4g/l yeast extract; 4g/l glucose). This culture was used to inoculate (2%) a 250 mL shake flask containing 50 mL of the same medium to which was added 300mg of the maize extract produced in example (a) above. This gave an equivalent to 2g L^{-1} ferulic acid and 0.18g L^{-1} coumaric acid substrates. The mixture was agitated at 500 rpm at 30° with a dissolved oxygen level of 60% of saturation. Substrate and product concentrations were measured by hplc as the following: 16 hours, ferulic acid 0.68g L^{-1}, vanillic acid 1g L^{-1}, coumaric acid 0.08g L^{-1}, 4-hydroxybenzoic acid 0.05g L^{-1}, 3,4-dihydroxybenzoic acid 0.02g L^{-1}; 18 hours, ferulic acid 0.14g L^{-1}, vanillic acid 1.54g L^{-1}, coumaric acid 0.035g L^{-1}, 4-hydroxybenzoic acid 0.08g L^{-1}, 3,4-dihydroxybenzoic acid 0.05g L^{-1}.

The product mixture can undergo further biotransformation, e.g. with reduction of $-CO_2H$ groups to $-CH_2OH$ and/or -CHO e.g. using Zygorhynchus moelleri or Micromucor isabellinus (see below). Thus the vanillic acid may be converted into a mixture of vanillin and vanillyl alcohol, in variable proportions. Likewise the 4-hydroxybenzoic acid may be converted into a mixture of 4-hydroxybenzaldehyde and 4-hydroxybenzyl alcohol. The minor acid components may undergo corresponding reductions. Thus the end product is a complex mixture of odoriferous compounds, principally of vanilla type. The proportion of aldehydes (such as vanillin) can be increased by biotransformation of the alcohols in the mixture, e.g. using Brevundimonas vesicularis.

Example 1C - Production of vanillic acid from Maize Fibre

Maize fibre (1kg) was subjected to citric acid hydrolysis (5L, 2% solution) followed by pressing and washing of the maize solids to give approximately 5 litres of maize liquor as described in (c) above. The pH of the liquor was adjusted to pH5.8 using 10M sodium hydroxide. A 2L aliquot of the above liquor was transferred into a 5L working volume fermenter and heated to 100°C for 1 minute. The remaining liquor was also heat-treated and stored separately. The fermenter was inoculated with 200 ml of a culture of *Penicillium chrysogenum* (Zyl 860) grown on pH5.8 maize liquor for 30 hours in a 1 litre conical flask (28°C shaking at 250 rpm). The fermenter contents were grown for 15 hours at 28°C while controlling the dissolved oxygen concentration at 30% of saturation. The pH of the culture was not controlled but remained unchanged throughout the incubation period. After 15 hours the volume of the fermenter contents was increased initially to 3.5L and then to 5L after a further 7 hours incubation. Incubation was continued as described previously for a further 3 days after which time the fermenter contents were assayed by hplc as described above. The maize liquor was shown to contain $1.2gl^{-1}$ vanillic acid and only a trace quantity of ferulic acid. Fungal biomass was separated from the maize liquor by a single pressing of the fermenter contents through a wine press to give a clarified aqueous product.

Whereas P.chrysogenum (Zyl 860) is the currently preferred strain, Aspergillus flavus (Zyl 714) and A.niger (Zyl 759) are also usable.

Vanillic acid can be recovered from aqueous solution by extraction into ethyl acetate followed by either evaporation of the solvent to dryness or by precipitation of vanillic acid from the solvent by the addition of hexane. For example, a culture broth (900 ml) comprising 567 mg vanillic acid was acidified to pH 3.0 and extracted three times with ethyl acetate (1x400ml, 2x200ml). The solvent extracts were combined, assayed by hplc as described above and found to contain 485mg vanillic acid (86%recovery).

An aliquot (200ml) was taken from the combined ethyl acetate extracts and the volume reduced to approximately 10ml by evaporation. Hexane (40ml) was added slowly to this concentrated extract, accompanied by constant mixing, the precipitated material was recovered by filtration and dried to yield 140mg of pale yellow solid comprising 115mg vanillic acid (82% purity). Recovery from the solvent was 88%, therefore, giving an overall recovery of 76% from the original culture broth.

Example 1D - Production of vanillin from maize fibre

The nutrient content of the clarified liquor from Example 1C was enhanced for growth of *Micromucor isabellinus* by the addition of nutrients as described in Example 4 below. No further vanillic acid was added to the liquor. A culture of *Micromucor isabellinus* (Zyl 849) was grown at 30°C for 3 days on pH 5.8 maize liquor (described in Example 1C) solidified with 1.5% agar. This culture was used to inoculate 20ml of the enhanced clarified liquor described above contained in a

100 ml conical flask. The flask contents were incubated at 30°C with shaking at 250 rpm for 24 hours, prior to being used to inoculate (2.5%) a second identical flask. Incubation conditions were as described previously. The concentrations of vanillic acid and vanillin in solution throughout the process were assayed by hplc as described above. After 24 hours incubation the pH of the liquor was slowly reduced over approximately 1.5 hours from pH5.2 to pH3.7. After 2.25 hours post onset of the pH reduction, $0.02gL^{-1}$ vanillin was detected in solution. After 4.5 hours the vanillin concentration had increased to $0.12gL^{-1}$. At 7.5 hours the vanillin and vanillic acid concentrations were $0.275gL^{-1}$ and $0.81gL^{-1}$ respectively. After 13 hours incubation the vanillic acid concentration had decreased to $0.45gL^{-1}$ and the concentration of vanillin had reached a maximum at $0.35gL^{-1}$.

B) Vanillin from vanillic acid via vanillyl alcohol

`One pot' bioconversions of vanillic acid to vanillin are known, but they generally show low yields and low conversion rates and/or low product concentrations (e.g. EP 453368, FR 2724394). We have found that Zygorhynchus moelleri can be used to produce very high concentrations of vanillyl alcohol (e.g. >5g/l) from vanillic acid. Vanillyl alcohol can be efficiently oxidised to vanillin, e.g. by means of Brevundimonas vesicularis.

Example 2: vanillyl alcohol from vanillic acid

A culture of Zygorhynchus moelleri (Zyl 851) grown on yeast malt agar was used to inoculate a 250mL starter culture flask containing 50mL medium, (20g glucose; 5g $(NH_4)_2SO_4$; 2g

NaCl; $MgSO_4$ 0.22g/L; $CaCl_2$ 0.015g/L, 10mL; trace element solution, 1mL; vitamins solution 10mL; made up to 1 litre with pH6.0 phosphate buffer, 0.2M), containing 2g/L vanillic acid, which was incubated at 30°C with shaking at 200rpm for 24 hours. This starter culture was added to 5 litres of the same medium in a fermenter except that the medium components were dissolved in deionised water and also containing 2g/L vanillic acid. This was stirred at pH 5.2, (30°C) for 24 hours after which the pH was altered to 3.5 over 1 hour, and the temperature kept at 30°C. Dissolved oxygen was maintained at 70% of saturation throughout the process. Assay was by hplc. Prior to the pH being allowed to drop (at the 24 hour stage), the amount of vanillic acid present in the system had not dropped and no products were seen. As the reaction proceeded, various amounts of substrate and nutrients were added as follows: 31.75 hours, 10g vanillic acid and 25g glucose; 47.5 hours, 50g glucose; 55 hours, 20g vanillic acid. Products were measured as being the following: 31.75 hours; vanillic acid, 0.26g/L, vanillyl alcohol, 0.3g/L; 47.5 hours, vanillic acid, 1.17g/L, vanillin, 0.1g/L, vanillyl alcohol, 2.38g/L; 55 hours, vanillic acid, 0.64g/L, vanillin, 0.06g/L, vanillyl alcohol, 3.5g/L; 5 days, vanillic acid, 1.21g/L, vanillin, 0.05g/L, vanillyl alcohol, 6.6g/L; after this stage no further product accumulation was observed. It is also evident that at pH 5.2 no substrate was converted into any products and the bioconversion only started when the pH was allowed to drop to pH 3.5.

Example 3: vanillin from vanillyl alcohol

Sterile nutrient broth (No 2) (50 mL) in a 250 mL shake flask was inoculated with <u>Brevundimonas vesicularis</u> (Zyl 295) and incubated at 30°C with shaking at 200rpm. After 24 hours the addition of 50mg of vanillyl alcohol was followed by the flask being monitored regularly by hplc for both vanillyl alcohol and vanillin. Up to 71 hours post inoculation the amount of vanillyl alcohol decreased to around 10% of the original level and the amount of vanillin increased to approximately 90% molar conversion from substrate. Identification of the structure of the product was confirmed by nmr spectroscopy.

If the vanillyl alcohol was added at the time of inoculation of the flask then the amount of substrate did not start to decrease until after 16 hours incubation when vanillin was first detected. The overall conversions closely followed those seen when substrate was added after 24 hours.

C) <u>Vanillin from vanillic acid</u>

As mentioned above, the biotransformation of vanillic acid into vanillin is known, but the yields of known methods are poor, making them unattractive in commercial terms. FR-A-2724394 discloses a process, using a basidiomycete, which gives fairly good yields and conversion rates in percentage terms, but whose absolute yields are low. The highest yield of vanillin produced in an example is 628mg/l.

We have found strains capable of affording vanillin at greater than 1g/l and usable in continuous production systems. Preferred microorganisms are strains of <u>Micromucor isabellinus</u> and <u>Aspergillus fumigatus</u>.

Example 4: production of vanillin by M.isabellinus

(a) A culture of Micromucor isabellinus (Zyl 849) grown on yeast malt agar was used to inoculate a 250 mL starter culture flask containing 50 mL medium (15g glucose; 5g $(NH_4)_2SO_4$; 2g K_2HPO_4; 0.2g NaCl; 0.2g $MgSO_4$; 0.015g $CaCl_2$, trace element solution, 1mL; vitamins solution 10mL; made up to 1 litre with deionised water) containing 2g/L vanillic acid which was incubated at 30°C with shaking at 200rpm for 16.5 hours. This starter culture was added to 5 litres of the same medium in a fermenter with vanillic acid added to a concentration of 1.5g L^{-1}. The fermenter contents were stirred at 30°C with the dissolved oxygen concentration being maintained at 70% of saturation throughout the process. Assay was by hplc as described above. As the reaction proceeded, additional amounts of vanillic acid were added as follows: 22.5 hours, 2.5g; 24 hours, 2.5g; 25.75 hours, 1g; 26.5 hours, 1g; 27.5 hours, 1g; 31 hours, 2.5g. Substrate and product concentrations were measured as being the following:: 22.5 hours, vanillic acid 0.6g L-$^{-1}$; vanillin 0.84 g L^{-1}; 24 hours, vanillic acid .1.36gL^{-1};vanillin 0.96 g L^{-1}; 25.75 hours, vanillic acid 1.15g L^{-1} ;vanillin 1.1g L^{-1}; 26.5 hours, vanillic acid 1.27g L^{-1} ;vanillin 1.17 g L^{-1}; 27.5 hours, vanillic acid 1.43g L^{-1};vanillin 1.33 gL^{-1}; 31 hours, vanillic acid 1.16gL^{-I};vanillin 1.6 gL^{-1}; 46 5 hours, vanillic acid 1.58g L^{-1} ;vanillin 1.70 g L^{-1}.

(b) A culture of Micromucor isabellinus (Zyl 849) grown on yeast malt agar was used to inoculate a 500 mL starter culture flask containing 100 mL medium (15g glucose; 5g $(NH_4)_2SO_4$; 2g K_2HPO_4; 0.2g NaCl;; 0.2g $MgSO_4$; 0.015g $CaCl_2$, trace element solution, 1mL; vitamins solution 10mL; made up to 1 litre with pH 6.0 phosphate buffer (0.2M)) containing 2g/L vanillic acid which was incubated at 30°C with shaking at 200rpm for 24 hours. This starter culture was added to 5 litres of the same medium in a fermenter with the medium components made up to 1 litre with deionised water and vanillic acid added to a concentration of 1.5g L^{-1}, immediately prior to inoculation. The fermenter contents were stirred at 30°C for 18 hours with the pH of the medium being maintained at pH 5.2 by the addition of 5M sodium hydroxide solution. After this time the medium pH was gradually reduced to 3.7 over 45 minutes by the addition of 5M HCl and temperature was maintained at 30°C. Dissolved oxygen concentration was maintained at 70% of saturation throughout the process. Assay was by hplc as described above. Prior to the pH being allowed to drop at the 18 hour stage, the amount of vanillic acid in the system was measured at 1.48 g L^{-1}; vanillin was detected in solution at 0.02 g L-1 . As the reaction proceeded various amounts of vanillic acid or glucose were added as follows: 19.25 hours, 50g glucose; 20.25 hours, 3g vanillic acid; 21.5 hours, 4g vanillic acid; 23 hours, 4g vanillic acid. Substrate and product concentrations were measured as being: 19.25 hours, vanillic acid 1.5 g $L-^{-1}$, vanillin 0.1 g $L-^{-1}$;: 20.25 hours, vanillic acid 1.16 g $L-^{-1}$, vanillin 0.36 g $L-^{-1}$;: 21.5 hours, vanillic acid 1.07 g $L-^{-1}$, vanillin 0.74 g $L-^{-1}$;: 23 hours, vanillic acid 1.06 g L^{-1}, vanillin 1.22 g $L-^{-1}$;: 26 hours, vanillic acid 0.83 g $L-^{-1}$, vanillin 1.96 g $L-^{-1}$.

Example 5: production of vanillin by A.fumigatus

A spore suspension of Aspergillus fumigatus (Zyl 747) was used to inoculate 5 litres of a minimal medium (ingredients described in Example 4(a), except for the addition of 3g $L-^{-1}$ vanillic acid rather than 1.5g L^{-1}). The fermenter contents were stirred without pH control at 30°C with the dissolved oxygen concentration being maintained at 60% of saturation. Assay was by hplc as described above. Substrate and product concentrations in the culture broth were as follows: 16 hours, vanillin 0.015g L^{-1}; 24 hours, vanillin 0.075g $L-^{-1}$; 40 hours, vanillin 0.69g $L-^{-1}$; 47 hours, vanillin 0.9 1g $L-^{-1}$; 48.5 hours, 0.98g $L-^{-1}$; 53.5 hours 1.02g$L-^{-1}$; 112 hours, vanillic acid 1.32gL^{-1}, vanillin 1.09gL^{-1}

D) Vanillin from ferulic acid

Example 6 Conversion of Ferulic acid to Vanillin by Zyl 581 an NTG derived mutant of Zyl503

Background

ZYL503 converts ferulic acid (FA) to vanillin acid (VA) via the intermediate vanillin. The rate-limiting step in this reaction is FA to vanillin, therefore accumulation of vanillin in culture media is not observed. Basd on the NTG mutation method described below a ZYL503 mutant has been produced that accumulates vanillin in culture media when supplied with FA.

(a) NTG Mutagenesis of ZYL503

ZYL503 was grown to mid-exponential phase in 10ml nutrient broth, harvested, washed once in 100mM sodium citrate buffer (pH 5.5), resuspended in 10ml of this buffer containing 0.1g l^{-1} NTG, and incubated at room temperature. At five minute intervals 0.5ml aliquots were taken, washed twice in 50mM potassium phosphate buffer, and resuspended in 1ml of this buffer. These samples were then diluted in 50mM phosphate buffer and spread onto a minimal medium agar (pH7, 0.4M phosphate buffered), that contained 20g l^{-1} glucose or 1 g l^{-1} vanillin plus 2 g l^{-1} yeast extract and 2 g l^{-1} ferulic acid. Plates were incubated at 30°C for 24-48h. Resultant colonies unable to grow on vanillin plates were selected. Zyl 581 was obtained based on this procedure.

(b) Conversion of commercial ferulic acid (free acid) to vanillin by Zyl 581

A preculture of Zyl 581 was grown in a 250 ml shake flask containing 50ml minimal medium (g l^{-1}: $(NH_4)_2SO_4$,5;K_2HPO_4,2;NaCl,0.2; Yeast extract, 0.2, glucose, 20: 10ml of a solution containing 0.1 M $MgSO_4$/0.01M $CaCl_2$; 10ml vitamin solution and 1ml trace elements solution at 200rpm, 30°C for 24h. This culture was then used to inoculate a bioreactor filled with 1.2 l of the same medium with pH control at 8.0 using 2M NaOH, oxygen control at 70% with a stirrer speed cascade of 100-400rpm air flow 1.3 vvm. Prior to inoculation 2 g l^{-1} ferulic acid (Lancaster, 99%) was added as the fermentation proceeded as indicated in the table below. An identical reactor was also set up with pH control at 8.5. Reaction products were quantified by HPLC.

pH 8.0

Time (h)	FA (g l^{-1})	VA (g l^{-1})	Vanillin (g l^{-1})	FA added (g l^{-1})
0	1.8881	0.000	0.000	
19.7	0.616	0.298	0.545	
21.2				1.380
21.7	1.953	0.433	0.460	
23.7	1.865	0.347	0.619	
25.7	1.806	0.798	0.260	
27.7	1.786	0.912	0.182	
43.4	1.105	1.039	0.278	
48.0	0.387	1.245	0.577	

pH 8.5

Time (h)	FA (g l^{-1})	VA (g l^{-1})	Vanillin (g l^{-1})	FA added (g l^{-1})
0	1.891	0.000	0.000	
19.7	0.520	0.000	0.545	
21.2				1.450
21.7	1.802	0.000	1.161	
23.7	1.380	0.000	1.418	
25.7	1.074	0.025	1.475	
26.0				0.970
27.7	1.829	0.038	1.521	

Time (h)	FA (g l^{-1})	VA (g l^{-1})	Vanillin (g l^{-1})	FA added (g l^{-1})
43.4	0.424	0.083	2.247	
44.8				1.540
48.0	1.871	0.115	2.193	

At pH8 vanillic acid was the predominant transformation product from ferulic acid while at pH 8.5 vanillin was the predominant product. After 43h at pH 8.5 the vanillin concentration was 2.247 g l^{-1} a molar yield of 73% for the ferulic acid consumed.

(c) <u>Conversion of sugar beet derived ferulic acid (sodium salt) to vanillin by Zyl581</u>

Ferulic acid obtained from Pentopan 500BG treated sugar beet liquor was extracted with butyl acetate, then back extracted into base to give the ferulic acid sodium salt (70.8% w/w ferulic acid as free acid).

Zyl581 was grown in a bioreactor as described above at pH 8.5 except that prior to inoculation 5.65 g/l sugar beet ferulic acid extract was added (4 g l^{-1} available ferulic acid present as sodium salt). After 40h the ferulic acid concentration in the reactor was 0.05g/l, vanillin acid 0.21 g/l and vanillin 1.69 g/l. This represents a molar yield for vanillin of 54%. 72% of the ferulic acid added could be accounted for as vanillic acid, vanillin or remaining ferulic acid.

(d) <u>Recovery of Vanillin from spent culture broth</u>

The culture broth from the Zyl581 fermentation was taken, cells removed by centrifugation, the broth adjusted to pH 7.5, overlaid with an equal volume of butyl acetate and stirred overnight. The butyl acetate was evaporated under vacuum and the resultant solid washed with 10ml hexane, recovered by filtration and dried at 40°C overnight.

400mg of the extracted solid from the Zyl581 fermentation (95% w/w vanillin by HPLC), was purified using the following procedure. The product was dissolved in 10ml diethyl ether to which 40 mg of silica (70-200μm) was added. The ether was evaporated leaving the solid adsorbed to the silica. The silica was washed with 500ml of a 2% (v/v) solution of ethyl acetate in petroleum ether (60-80 fraction). This solution was filtered through a glass sinter and evaporated under vacuum to yield a white amorphous solid containing greater than 99% w/w vanillin (277mg).

E) <u>In situ product removal ("ISPR")</u>

The principle involves carrying out a biotransformation in an aqueous phase which is in contact with, or is contacted with, an immiscible phase into which a product can pass (exclusively or preferentially). Possible advantages include a) the protection of a product from further reaction in the aqueous phase; b) avoidance of inhibition of product formation by a microorganism by high product concentrations; c) enabling equilibrium reactions to convert a larger proportion of starting material into product; and d) ease of isolation of products. Furthermore ISPR can assist in the development of continuous systems.

ISPR is readily applied to systems where a polar substrate (e.g. a carboxylic acid such as ferulic or vanillic acid) is converted into a less polar product, which will be preferentially extracted by a nonpolar solvent such as a plant oil material, preferably food grade. Polar byproducts will also tend to stay in the aqueous phase.

Examples 7 and 8 correspond to Examples 4 and 5 but make use of ISPR.

Depending on the system, the immiscible phase may contact the aqueous phase while biotransformation is proceeding therein, or it may contact portions of the aqueous phase which have been withdrawn (temporarily) from the bioreactor. The withdrawn phase may be treated (e.g. by adjustment of pH) to facilitate extraction by the immiscible phase.

Example 7: production of vanillin by M.isabellinus with ISPR

A 5 litre culture of Micromucor isabellinus (Zyl 849) was grown in a fermenter as described in Example 4b. The fermenter contents were stirred at 30°C for 20 hours with the pH of the medium maintained at 5.2 by the addition of 5M sodium hydroxide solution. After this time the medium pH was gradually reduced to 3.8 over 45 minutes by the addition of 5M HCl and was maintained at this pH thereafter; temperature was maintained at 30°C. Dissolved oxygen concentration was maintained at 70% of saturation throughout the process. Assay was by hplc as described above. The progress of the reaction was monitored until the concentration of vanillin in the culture broth reached 0.96 g L^{-1} . Throughout this period vanillic acid was added to the medium to maintain a concentration of 1.5 g L^{-1}. At this time an external vanillin extraction system was activated as follows: culture medium was continuously pumped from the fermenter through a filtration device such that the biomass was retained within the fermenter; culture medium exiting the fermenter was heated to 60°C and adjusted to pH 6.5 by the addition of 10M sodium hydroxide solution; this medium was fed into an extraction vessel containing 5litres of sunflower oil; the aqueous phase (maintained at 1 litre volume) and the oil phase were stirred vigorously by the use of an overhead stirrer to effect continuous selective extraction of vanillin from the culture medium into the oil phase; vanillic acid did not extract into the oil and remained entirely in the aqueous phase; this aqueous phase was continually pumped back into the fermenter. Throughout the process, the volume in the fermenter and the volume in the extraction vessel remained relatively constant. The concentration of vanillic acid in the fermenter was maintained at approximately 1.5 g L^{-1} and the concentration of vanillin in the fermenter was maintained at a maximum of 1.5 g L^{-1}. Using this continuous external extraction facility the 5 litre culture of <u>Micromucor isabellinus</u> produced 18.9g vanillin over an operational period of approximately 20 hours. Vanillin can be recovered from sunflower oil by extraction into water, or alcohol, e.g. methanol or ethanol, or into an alcohol/water mixture. A suitable mixed solvent is 80% ethanol and 20% water. After evaporation of the extraction solvent, vanillin can be further purified by traditional techniques such as recrystallisation, sublimation etc.

Example 8: production of vanillin by A.fumigatus with ISPR

A sterile glass column (60mL volume) packed with a stainless steel support was filled with a minimal medium (ingredients described in Example 4) containing 2g L^{-1} vanillic acid and inoculated with spores of Aspergillus fumigatus (Zyl 747). Air was pumped into the base of the column in order to aerate the system and effect efficient mixing. The column was allowed to stand at room temperature (22°C) for 70 hours. After this time substantial growth of the fungus had occurred and all had adhered to the stainless steel support material. The concentration of vanillin in solution was measured as $0.65gL^{-1}$. Maintaining aeration as described above, the contents of the column were continuously pumped out of the column into a separate extraction vessel containing 500mL sunflower oil and 200mL of the same minimal medium described previously. In addition, 1g vanillic acid was added to the aqueous phase. The contents of the extraction vessel were mixed thoroughly and the aqueous phase continuously pumped back through the column. Incubation of the column contents at approximately 30°C was achieved by jacketing the column with silicon tubing and pumping water through at a temperature of 34°C. The concentration of vanillin in both the oil and aqueous phase inside the extraction vessel was assayed (hplc, as described above) at intervals over an eight day period. Results, expressed as total vanillin yield from the system, were as follows: 24 hours, 113mg; 48 hours, 140mg; 72 hours, 269mg; 96 hours, 385mg; 168 hours, 597mg; 192 hours, 566mg.

The suitability of the system of example 8 for long-term continuous use is demonstrated by Example 9.

Example 9: continuous production of vanillin using ISPR

A spore suspension of Aspergillus fumigatus (Zyl 747) was used to inoculate 3 litres of a minimal medium (ingredients described in Example 9 except for the addition of 3.3g L^{-1} vanillic acid rather than 1.5g) in a fermenter (5L working volume). The fermenter contents were stirred without pH control at 30°C with the dissolved oxygen concentration being maintained at 60% of saturation throughout the process. Assay was by hplc as described above. After 24 hours incubation, 0.15g L^{-1} vanillin was detected in solution. At this time 2 litres of sunflower oil containing 0.5g L^{-1} vanillic acid was added to the fermenter and incubation was continued as described previously. Over a period of 28 days, the oil phase in the fermenter was removed at frequent intervals and replaced with fresh oil containing 0.5g $L-^{-1}$ vanillic acid Vanillin production expressed as the total vanillin yield from the system was as follows:

Time (hours)	Vanillin yield (g)
48	0.64
72	1.43
96	2.71

168	4.60
192	5.12
216	5.58
240	6.20
264	6.87
336	9.16
360	10.32
384	11.50
408	12.25
432	12.80
504	13.80
575	14.50
665	16.26

After 665 hours the experiment was terminated; however, the fungus was still actively producing vanillin.

G) Use of *Pseudomonas putida* for vanillic acid production

Example 10 Conversion of ferulic acid to vanillic acid

A nutrient agar plate culture of *Pseudomonas putida* (zyl 503) was used a source of inoculum for 50 ml of growth medium (5 g ferulic acid; 20 g glucose; 2g KH_2PO_4; 5g $(NH_4)_2SO_4$; 0.2g NaCl; 0.22 g $MgSO_4$; 0.015g $CaCl_2$; 1 ml trace elements solution; 10 ml vitamins solution, made up to 1 litre with 0.2 M, pH 7.0 phosphate buffer) in a 250 ml conical shake flask. The culture was incubated at 30°C shaking at 250 rpm and assayed by hplc as described above. As the reaction proceeded, additional amounts of ferulic acid were added as follows: 24.5 hours 0.25 g; 48 hours 0.125 g; 72 hours 0.05 g; 90 hours

0.25 g; 96 hours 0.5 g. Substrate and product concentrations were measured as being the following: 20.5 hours, ferulic acid 2.71 gl^{-1} ,vanillic acid 2.08 gl^{-1} ;24.5 hours, ferulic acid 1.51 gl^{-1}, vanillic acid 3.01 gl^{-1}; 48 hours, ferulic acid 2.4 gl^{-1} ,vanillic acid 7.0 gL^{-1}; 72 hours, ferulic acid 2.08 gl^{-1}, vanillic acid 9.5 gL^{-1}; 96 hours ferulic acid 4.47 gL^{-1}, vanillic acid 12.11g L^{-1}; 160 hours, ferulic acid 4.45 gL^{-1}, vanillic acid 19.05 gL^{-1}.

<u>Example 11 Production of vanillic acid from maize fibre</u>

To 30 g maize fibre in a 250 ml conical flask was added 100 ml of 8% w/v citric acid solution. The flask contents were heated at 85°C with efficient mixing for 16 hours. After this time, the stirred maize fibre suspension was neutralised by the dropwise addition of sodium hydroxide solution (10M). A hydrolytic enzyme preparation (500μl, Biofeed Plus, Novo Nordisk) was added to the suspension and the whole incubated at 45°C with continued mixing for 24 hours. Analysis was by hplc as described above. After 24 hours 3.3 gl^{-1} ferulic acid was detected in solution.

A culture of *Pseudomonas putida* (zyl 503) grown on nutrient agar was used to inoculate a 250 ml shake flask containing 50 ml of minimal salts medium (4 g ferulic acid; 20 g glucose; 5 g $(NH_4)_2SO_4$; 0.2 g NaCl; 2 g K_2HPO_4; 0.22 g $MgSO_4$; 0.015 g

$CaCl_2$; 1 ml trace elements solution; 10 ml vitamins solution made up to 1 litre with pH 7.0 phosphate buffer 0.2 M. The flask contents were incubated at 30°C shaking at 250 rpm for 24 hours. After this time the culture was harvested by centrifugation (4000 x g, 20 minutes), washed once with 0.2 M pH 7.0 phosphate buffer to remove residual non maize derived ferulic or vanillic acid and finally resuspended in 2 ml of the same buffer (x 25 concentration). This concentrated cell suspension was added to a 20 ml aliquot of the hydrolysed maize suspension described above contained in a 100 ml conical flask. The concentration of ferulic acid in solution was measured as 2.8 gl^{-1} prior to incubation at 30°C with shaking at 250 rpm. The concentration of vanillic acid present throughout the incubation period was measured as being the following: 4 hours, 0.11 gl^{-1}; 24.5 hours, 1.44 gL^{-1}; 29.5 hours, 1.98 gL^{-1}; 31 hours, 2.0 gL^{-1}.

H) __Transformations of other substrates__

The conversion of ferulic acid into vanillic acid as in example 1 is the conversion of a cinnamic acid ($AR-CH=CH-CO_2H$) to a benzoic acid ($AR-CO_2H$). This can be applied to other cinnamic acids, e.g. coumaric acid (4-hydroxycinnamic acid) and caffeic acid (3,4-dihydroxycinnamic acid).

__Example 12: 4-hydroxybenzoic acid from coumaric acid__

A seed stage culture of __Rhodotorula glutinis__ (Zyl 702) was grown for 24 hours at 30°C with shaking at 200 rpm in a 250 mL shake flask containing 50 mL of a minimal medium (as defined in Example 1). This culture was used to inoculate (2%) a 250 mL shake flask containing 50 mL of the same minimal medium with the addition of 200 mg coumaric acid to give a final concentration of 4 gL^{-1}. Incubation conditions were as described previously and assay was by hplc as described above. Substrate and product concentrations were measured as the following: 18 hours, coumaric acid 1.75 gL^{-1}, 4-hydroxybenzoic acid 1.28g L^{-1}, 3,4 dihydroxybenzoic acid 0.27 gL^{-1}; 22 hours, coumaric acid 0.36 gL^{-1}, 4-hydroxybenzoic acid 1.92 gL^{-1}, 3,4-dihydroxybenzoic acid 0.44 gL^{-1}; 23 hours, coumaric acid 0.12 gL^{-1}, 4-hydroxybenzoic acid 2.10 gL^{-1}, 3,4-dihydroxybenzoic acid 0.47 gL^{-1}.

The conversion of vanillic acid to vanillin as in example 4 is the conversion of a hydroxybenzoic acid to a hydroxybenzaldehyde. This can be applied to other benzoic acids, particularly hydroxybenzoic acids, e.g. 4-hydroxybenzoic acid (as produced in Example 12) or 3,4-dihydroxybenzoic acid (protocatechuic acid).

Example 13: conversion of 4-hydroxybenzoic acid to 4-hydroxybenzaldehyde and 4-hydroxybenzyl alcohol

A culture of *Zygorhynchus moelleri* (Zyl 851), grown on yeast malt agar, was used to inoculate a 250 mL conical flask containing 42 mL of culture medium containing 100 mg 4-hydroxybenzoic acid. The culture broth was incubated at 30°C with shaking at 200 rpm. The progress of the reaction was assayed by hplc as described above. Substrate and product concentrations were measured as being the following: 24 hours, 4-hydroxybenzoic acid 2.26g L^{-1}, 4-hydroxybenzaldehyde trace amount, 4-hydroxybenzyl alcohol trace amount; 42 hours, 4-hydroxybenzoic acid 1.06g L^{-1}, 4-hydroxybenzaldehyde 0.53g

L^{-1}, 4-hydroxybenzyl alcohol 0.55g L^{-1}; 66 hours, 4-hydroxybenzoic acid 0.1 g L^{-1}, 4-hydroxybenzaldehyde trace amount, 4-hydroxybenzyl alcohol 2.6g L^{-1}.

Example 14; conversion of 4-hydroxybenzoic acid to 4-hydroxybenzaldehyde

A culture of *Trichoderma koningii* (Zyl 751) grown on yeast malt agar, was used to inoculate 50 mL of a minimal medium (as defined in Example 4a) in a 250 mL conical flask. Prior to inoculation, 150mg (3 gL^{-1}) 4-hydroxybenzoic acid was added to the medium followed by incubation of the whole at 30°C with shaking at 200 rpm. Assay was by hplc and tlc as described above. After approximately 30 hours incubation, hplc analysis detected 0.3 gL^{-1} 4-hydroxybenzaldehyde in solution. This observation was further supported by tlc analysis which revealed the presence of material at R_f 0.59 consistent with a reference sample of 4-hydroxybenzaldehyde. The observed product also gave a positive colour reaction with dinitrophenylhydrazine solution.

Example 15: conversion of 3,4-dihydroxybenzoic acid to 3,4-dihydroxybenzaldehyde

A culture of *Zygorhynchus moelleri* (Zyl 851), grown on yeast malt agar, was used in inoculate a 250 mL conical flask containing 50 mL of culture medium containing 100mg vanillic acid. The culture broth was incubated at 30°C with shaking at 200 rpm. The progress of the reaction was assayed by hplc as described above. After 42 hours incubation, approximately 50% of the vanillic acid had been converted to vanillyl alcohol. At this time, 100mg of 3,4-dihydroxybenzoic acid (e.g. from Example 12, or extracted from onion skins) was added to the culture and incubation continued. After a further 6 hours incubation a new product corresponding to 3,4-dihydroxybenzaldehyde was detected at a concentration of approximately 0.20g L^{-1}. After 24 hours this product concentration had increased slightly to approximately 0.025g L^{-1}. After 48 hours the 3,4-dihydroxybenzaldehyde had been lost from the solution.

Example 16; Conversion of benzoic acid to benzaldehyde

In a 1 litre flask, 200 mL of medium, (50g glucose; 5g $(NH_4)_2SO_4)_4$;2g K_2HPO_4;0.2g NaCl;0.22g $MgSO_4$; 0.015g $CaCl_2$ 1ml trace element solution, 1mL; 10ml vitamins solution; made up to 1 litre with deionised water), was inoculated with a 10μL loopful of spores of Trichoderma koningii (Zyl 751) after the addition of 50mg of benzoic acid. This was incubated at 30°C with shaking at 200rpm. By hplc analysis, benzaldehyde was first detected in the culture broth at 25 hours incubation. Over the next 5 hours benzaldehyde concentration rose to 0.165g/L; at this time a concentration of 0.1g benzyl alcohol was also present in the solution.

The conversion of vanillyl alcohol into the aldehyde in example 3 can also be applied to other benzyl alcohols, e.g. 4-hydroxybenzyl alcohol as produced in example 14.

Example 17: conversion of 4-hydroxybenzyl alcohol to 4-hydroxybenzaldehyde

The methodology described in Example 17 was followed but at the 24 hour stage of growth of the organism 4-hydroxybenzyl alcohol was added to the flask to give a final concentration of 1 mg/mL. Monitoring the progress of the reaction by hplc showed that the amount of substrate dropped to 10% of the original after a further 28 hours at which stage 4-hydroxybenzaldehyde had reached a concentration of 0.37 mg/mL.

NB the yields of the aldehydes in examples 14 - 17 could doubtless be improved by the use of ISPR.

I) Selective screening for organisms producing aldehydes from carboxylic acids

i) Preparation of agar plates

Minimal salts agar: 20g glucose; 5g $(NH_4)_2SO_4$;2g K_2HPO_4;0.2g NaCl; 0.22g $MgSO_4$; 0.015g $CaCl_2$; 10ml trace element solution; 1ml vitamins solution; 20g agar and 2g vanillic or ferulic acid made up to 1 litre with either deionised water (fungal isolation) or with 0.2M, pH 7.0, sodium phosphate buffer (bacterial isolation).

Filter paper (Whatman No.1) was cut into discs with a diameter of 90 mm and sterilised by autoclaving. Single discs were placed into 90mm sterile petri dishes prior to the pouring of a minimal salts agar described above.

ii) Preparation of soil samples

To 2ml deionised water was added approximately 100mg soil. The resulting suspension was mixed thoroughly (vortex mixer); allowed to stand at room temperature (22°C) for 1 hour followed by further mixing to distribute suspended material. The macroscopic solids were allowed to settle for approximately 10 minutes and the supernatant (100μl) applied to the prepared minimal salts agar plates using a spread plate technique. Plates were incubated at 28°C until colony development was observed (approx. 5 days)

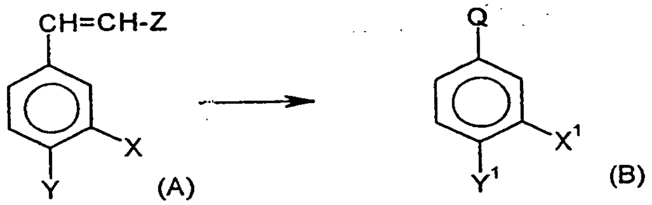
iii) Selective visualisation of aldehyde producing strains

In order to visualise vanillic acid or ferulic acid biotransformation products the following procedure was followed:

Agar was lifted from the base of each petri dish by inserting a spatula beneath the filter paper disc, followed by the injection of 1ml of dinitrophenyl hydrazine (DNP) solution (0.4% DNP in 2M HCl). The agar was replaced in the dish and the DNP solution allowed to permeate through the agar. Colonies producing aldehyde products were visualised by the presence of an orange/red zone surrounding the colony against a pale yellow background. Colonies producing alcohol products were visualised by the presence of a dark yellow zone surrounding the colony, against the pale yellow background.

CLAIMS:

1. A method of converting a first composition comprising one or more species of formula (A) into a second composition comprising one or more species of formula (B):



where X, Y, X' and Y' are independently selected from H, OH and OMe; Z is CO_2H, CO_2*carb* (where *carb* represents a carbohydrate residue), CHO or CH_2OH, and Q is CHO, CO_2H or CH_2OH, said method comprising treating said first composition with one or more microorganisms under conditions such that (A) is converted into (B); said microorganism(s) being selected, from (a) *Pseudomonas putida*; (b) *Rhodotorula* species and other yeasts capable of converted ferulic acid into vanillic acid;(c) microorganisms possessing both ferulic acid esterase activity and intra-sidechain cleavage activity such that they are capable of converting ferulic acid glycosides into vanillin and/or vanillic acid; and (d) *Micromucor isabellinus* or *Aspergillus fumigatus* strains capable of converting vanillin acid into vanillin.

2. A method according to claim 1 wherein Y and Y' are OH.

3. A method according to claim 1 or claim 2 wherein Z is CO_2H or CO_2*carb*.

4. A method according to claim 1, 2 or 3 wherein said first composition comprises ferulic acid or one or more ferulic acid esters and said second composition comprises vanillic acid and/or vanillyl alcohol and/or vanillin.

5. A method according to Claim 4 wherein said first composition comprises ferulic acid and said second composition comprises vanillic acid and said microorganism is selected from (a) and (b)

6. A method accroding to Claim 5 wherein said microorganism is *Rhodotorula glutinis*.

7. A method according to Claim 5 wherein said microorganism is *Pseudomonas putida* NCIMB40988 or *Rhodotorula glutinis* IMI379896.

8. A method according to Claim 4 wherein said first composition comprises a ferulic acid ester and said microorganism is selected from (c).

9. A method according to Claim 8 wherein said microorganism is selected from *Penicillium* and *Aspergillus* species.

10. A method according to Claim 9 wherein said microorganism is selected from *P.chrysogenum, A.niger* and *A.flavus*.

11. A method according to Claim 10 wherein said microorganism is selected from P.chrysogenum IMI379901, A.flavus IMI379895 and A.niger IMI379904.

12. A process according to any of claims 8 to 11 wherein said ferulic acid ester is provided in the form of plant material, said microorganism acting directly on said plant material.

13. A method according to any preceding Claim wherein said treatment of the first composition affords a second composition comprising vanillic acid, and said second composition is treated with one or more further microorganisms for converting said vanillic acid into vanillin.

14. A method according to Claim 13 wherein said second composition is treated with a second microorganism for converting said vanillic acid into vanillyl alcohol; and then with a third microorganism for converting said vanillyl alcohol into vanillin.

15. A method according to Claim 14 wherein said second microorganism is *Zygorhynchus moelleri*.

16. A method according to Claim 14 wherein said second micoorganism is *Zygorhynchus moelleri* IMI379899.

17. A method according to Claim 14, 15 or 16 wherein said third microorganism in *Brevundimonas vesicularis*.

18. A method according to Claim 17 wherein said third microorganism in *Brevundimonas vesicularis* NCIMB40987.

19. A method according to Claim 11 wherein said second composition is treated with *Aspergillus fumigatus* or *Micromucor isabellinus* for converting vanillic acid into vanillin.

20. A method according to Claim 17 wherein said second composition is treated with *A.fumigatus* IMI379902 or *M.isabellinus* IMI379893.

21. A method according to Claim 4 wherein said first composition is treated with a strain of *Pseudomonas* under conditions such that vanillin accumulates.

22. A method according to Claim 21 wherein said strain is a *Pseudomonas putida*.

23. A method according to Claim 21 wherein said strain is *Pseudomonas putida* IMI382568.

24. A method according to Claim 21, 22 or 23 wherein said strain is capable of producing both vanillic acid and vanillin from ferulic acid, the ratio thereof being pH-dependent; and wherein a pH is selected and maintained which relatively favours accumulation of vanillin.

25. A method according to any preceding claim wherein said second composition contains at least one desired component which is susceptible to further transformation; and wherein said transformation of said first composition is effected in an aqueous phase which is contacted with an organic phase which extracts said at least one desired component.

26. A method according to any preceding claim wherein said first composition contains ferulic acid and at least one further compound of the formula (A) where Z is neither CO_2H nor CO_2 *carb*, and wherein said at least one further compound also undergoes transformation.

27. A mixture resulting from the method of Claim 26 which includes vanillin.

28. A method according to any of claims 1 to 26 including a preliminary step of liberating said first composition from plant material.

29. A method according to any of Claims 1 to 26 including a preliminary step of obtaining said first composition comprising ferulic acid from a plant material by a process comprising:

(a) treating the plant material to produce a solution containing a ferulic acid ester; and

(b) treating said solution with an enzyme composition having ferulic acid esterase activity under conditions such that ferulic acid esters are converted into ferulic acid.

30. A method according to claim 28 or 29 wherein said plant material is selected from maize, wheat, sugar beet and rice materials.

31. A method according to claim 30 wherein said plant material comprises fibre, bran or straw.

32. A method according to Claim 29, 30 or 31 wherein in step (a) the plant material is treated with a solution containing citric acid or sodium bicarbonate.

33. A method according to claim 32 wherein said plant material is treated in the temperature range 50-250°.

34. A method according to Claim 29 wherein the plant material comprises sugar beet fibre and step (a) involves heating in water.

35. A method according to any of Claims 29 to 34 wherein step (b) employs an enzyme derived from a species of *Aspergillus* or *Humicola insolens*.

36. A method according to Claim 35 wherein the enzyme derived from *Humicola insolens* and treatment is effected at substantially in the pH range 6-7.

37. A method of obtaining a strain of microorganism for use in the method of any of Claims 1 to 26 or 28 to 36 comprising screening a multiplicity of colonies by means of a reagent suitable for detecting aldehydes.

38. A method according to Claim 37 wherein the multiplicity of colonies are obtained by mutation.

39. A method according to any of Claims 1 to 26 or 28 to 36 including a preliminary step of obtaining a microorganism for use therein by the method of Claim 37 or Claim 38.

40. *Pseudomonas putida* NCIMB40988 and mutants thereof capable of converting ferulic acid into vanillic acid.

41. *Rhodotorula glutinis* IMI 379896 and mutants thereof capable of converting ferulic acid into vanillic acid.

42. *Penicillium chrysogenum* IMI379900 and mutants thereof capable of converting a ferulic acid ester into vanillic acid.

43. *Aspergillus flavus* IMI379895 and mutants thereof capable of converting a ferulic acid ester into vanillic acid.

44. *Aspergillus niger* IMI379904 and mutants thereof capable of converting a ferulic acid ester into vanillic acid.

45. *Zygorhynchus moelleri* IMI379899 and mutants thereof capable of converting vanillic acid into vanillyl alcohol and vanillin.

46. *Pseudomonas putida* IMI382568 and mutants thereof capable of converting ferulic acid into vanillin.

47. *Aspergillus fumigatus* IMI379902 and mutants thereof capable of converting vanillic acid into vanillin.

48. *Micromucor isabellinus* IMI379893 and mutants thereof capable of converting vanillic acid into vanillin.

49. *Brevundimonas vesicularis* NCIMB40987 and mutants thereof capable of converting vanillyl alcohol into vanillin.

50. *Trichoderma koningii* IMI 379903 and mutants thereof capable of converting 4-hydroxylbenzoic acid into 4-hydroxybenzaldehyde.

51. An extract or enzyme(s) of an organism defined in any of claims 40-50 possessing the activity specified for the organism.

52. Microbial conversion of coumaric acid into p-hydroxybenzoic acid using *Rhodotorula glutinis* or *Pseudomonas putida* or an extract or enzyme(s) therefrom.

53. Microbial conversion of p-hydroxybenzoic acid into p-hydroxybenzyl alcohol using *Zygorhynchus moelleri* or an extract or enzyme(s) therefrom.

54. Microbial conversion of p-hydroxybenzoic acid into p-hydroxybenzaldehyde using *Trichoderma koningii* or *Zygorhynchus moelleri* or an extract or enzyme(s) therefrom.

55. Microbial conversion of p-hydroxybenzyl alcohol into p-hydroxybenzaldehyde using *Brevundimonas vesicularis* or an extract or enzyme(s) therefrom.

56. Microbial conversion of 3,4-dihydroxybenzoic acid into 3,4-dihydroxy benzaldehyde using *Zygorhynchus moelleri* or an extract or enzyme(s) therefrom.

57. Microbial conversion of benzoic acid into benzaldehyde using *Trichoderma koningii* or an extract or enzyme(s) therefrom.

58. Microbial conversion of ferulic acid into vanillic acid using *Pseudomonas putida* or *Rhodotorula glutinis* or an extract or enzyme(s) therefrom.

59. Microbial conversion of a ferulic acid ester into vanillic acid using *Penicillium chrysogenum*, *Aspergillus niger* or *Aspergillus flavus* or an extract or enzyme(s) therefrom.

60. Microbial conversion of vanillic acid into vanillin using *Aspergillus fumigatus* or *Micromucor isabellinus* or an extract or enzyme(s) therefrom.

61. Microbial conversion of ferulic acid into vanillin using *Pseudomonas putida* or an extract or enzyme(s) therefrom.

62. Microbial conversion of vanillic acid into vanillyl alcohol using *Zygorhynchus moelleri* or an extract or enzyme(s) therefrom.

63. Microbial conversion of vanillyl alcohol into vanillin using *Brevundimonas vesicularis* or an extract or enzyme(s) therefrom.

☐ Generate Collection

L6: Entry 6 of 7 File: DWPI Nov 7, 1995

DERWENT-ACC-NO: 1996-015289
DERWENT-WEEK: 199602

TITLE: Determn. of decomposition by microorganisms of aromatic cpds. - by incubating microorganism with creosote and observing colour generation

PATENT-ASSIGNEE:

ASSIGNEE	CODE
CANON KK	CANO

~ background

PRIORITY-DATA: 1994JP-0089912 (April 27, 1994)

PATENT-FAMILY:

PUB-NO	PUB-DATE	LANGUAGE	PAGES	MAIN-IPC
JP 07289290 A	November 7, 1995		004	C12Q001/04

APPLICATION-DATA:

PUB-NO	APPL-DATE	APPL-NO	DESCRIPTOR
JP07289290A	April 27, 1994	1994JP-0089912	

INT-CL (IPC): C02 F 3/00; C12 N 1/00; C12 N 1/20; C12 Q 1/04; C12 Q 1/04; C12 R 1:40; C12 Q 1/04; C12 R 1:38; C12 N 1/00 ; C12 R 1:01; C12 N 1/20; C12 R 1:38

ABSTRACTED-PUB-NO: JP07289290A
BASIC-ABSTRACT:

Determn. of the activity of a microorganism in decomposition of aromatic cpds. and/or organic chlorine cpds. which comprises incubating the microorganism (specific: Pseudomonas cepacia, Pseudomonas putida) in the presence of creosote and determining the activity of the microorganism decomposing said aromatic cpds. (specific: phenol, toluene, o-, m- or p-cresol, vanillin, vanillic acid) and/or organic chlorine cpds. (specifically chlorinated ethylene; trichloroethylene, dichloroethylene) depending on a colour which is generated by decomposition of creosote, is new. Also claimed is a method for screening microorganisms decomposing aromatic cpds. and/or organic chlorine cpds. which comprises incubating several microorganisms in the presence of aromatic cpds. and/or organic chlorine cpds. to form a colony or colonies to screen one or more species of microorganisms, then contacting the colony or colonies with a creosote soln. to incubate, and determining a colour generated from creosote. Also claimed is a method for cleanup of environment which comprises dispersing a microorganism selected by the above screening method to environment which has been polluted by aromatic cpds. and/or organic chlorine cpds. USE/ADVANTAGE - The first method can be used in determining whether the microorganism tested has an activity decomposing pollution materials, aromatic cpds. and/or organic chlorine cpds. The 2nd method can be used in selecting an active one from a mixt. of microorganisms. Creosote generates a reddish brown colour which can be distinguished clearly and is maintained for a long period of time.

CHOSEN-DRAWING: Dwg.0/0

TITLE-TERMS: DETERMINE DECOMPOSE MICROORGANISM AROMATIC COMPOUND INCUBATE MICROORGANISM CREOSOTE OBSERVE COLOUR GENERATE

(19)日本国特許庁（ＪＰ）

(12)公開特許公報(A)

(11)特許出願公開番号

特開平7－289290

(43)公開日　平成7年(1995)11月7日

(51)Int.Cl.^{6}		識別記号	庁内整理番号	FI	技術表示箇所
C12Q	1/04		6807－4B		
C02F	3/00	G			
C12N	1/00	T	8828－4B		
	1/20	ZAB F	8828－4B		
//（C12Q	1/04				

審査請求　未請求　請求項の数12　OL　（全 9 頁）　最終頁に続く

(21)出願番号　特願平6－89912

(22)出願日　平成6年(1994)4月27日

(71)出願人　000001007
キヤノン株式会社
東京都大田区下丸子3丁目30番2号

(72)発明者　今村　剛士
東京都大田区下丸子3丁目30番2号　キヤノン株式会社内

(74)代理人　弁理士　若林　忠

(54)【発明の名称】　微生物分解活性確認方法、それを用いた微生物スクリーニング方法および環境浄化方法

(57)【要約】

【目的】　明瞭に識別でき、かつ安定しており長時間維持でき、また分解活性の発現段階に相応する呈色を得、もって、微生物の分解活性の有無の確認および微生物が高い分解活性を発現する段階の確認を確実に、かつ簡便に行う。

【構成】　クレオソートの存在下でシュードモナス属等に属する微生物を培養して該クレオソートを該微生物により分解し得られる呈色物質の生成を指標として、該微生物の有する置換ベンゼン分解活性または塩素化エチレン等の芳香族化合物および／または有機塩素化合物分解活性を確認する。

【特許請求の範囲】

【請求項１】　クレオソートの存在下で微生物を培養して該クレオソートを該微生物により分解し得られる呈色物質の生成を指標として、該微生物の有する芳香族化合物および／または有機塩素化合物分解活性を確認することを特徴とする微生物分解活性確認方法。

【請求項２】　請求項１において、クレオソートの濃度が、培地中１０～１０００ppmである微生物分解活性確認方法。

【請求項３】　請求項１または２において、確認する芳香族化合物分解活性が、置換ベンゼン分解活性である微生物分解活性確認方法。

【請求項４】　請求項３において、置換ベンゼン分解活性が、フェノール、トルエン、o-クレゾール、m-クレゾール、p-クレゾール、バニリンおよびバニリン酸から選ばれる一種以上を分解する活性である微生物分解活性確認方法。

【請求項５】　請求項１または２において、確認する有機塩素化合物分解活性が、塩素化エチレンである微生物分解活性確認方法。

【請求項６】　請求項５において、塩素化エチレン分解活性が、トリクロロエチレン、ジクロロエチレンのうちの少なくとも一種を分解する活性である微生物分解活性確認方法。

【請求項７】　請求項１乃至６のいずれか一項において、微生物が芳香族化合物を酸化分解できる微生物である微生物分解活性確認方法。

【請求項８】　請求項１乃至６のいずれか一項において、微生物が芳香族化合物資化揮発性有機塩素化合物分解菌である微生物分解活性確認方法。

【請求項９】　請求項７または８において、微生物がシュードモナス属に属する微生物である微生物分解活性確認方法。

【請求項１０】　請求項９において、微生物がシュードモナス・セパシアまたはシュードモナス・プチダである微生物分解活性確認方法。

【請求項１１】　芳香族化合物および／または有機塩素化合の存在下で複数の微生物を培養してコロニーを形成させて一種以上の微生物をスクリーニングし、次に、クレオソート含有溶液を該コロニーに接触させて培養し、該クレオソートを分解する微生物を呈色物質の生成を指標としてスクリーニングすることを特徴とする芳香族化合物および／または有機塩素化合物分解活性を有する微生物のスクリーニング方法。

【請求項１２】　クレオソートの存在下で微生物を培養して該クレオソートを該微生物により分解し、培養液がクレオソートの分解により呈色するのを確認した後、該培養液から該微生物を回収精製し、この微生物を、芳香族化合物および／または有機塩素化合物により汚染された環境に分散させ該環境を浄化することを特徴とする微生物による環境浄化方法。

【発明の詳細な説明】

【０００１】

【産業上の利用分野】本発明は、環境浄化分野や有用物質の微生物変換分野等における、芳香族化合物および／または有機塩素化合物の微生物分解活性確認方法に関し、特に、クレオソートを用いることにより、該分解活性確認の確実性を向上させ、かつ飛躍的に簡便化する微生物分解活性確認方法、それを用いた微生物スクリーニング方法および環境浄化方法に関する。

【０００２】

【従来の技術】これまで、フェノールやトルエン等の芳香族化合物に、酸素原子を付与する形で酸化を行うオキシゲナーゼ系或いはヒドロキシラーゼ系の酵素を持つ細菌類は環境中より多数単離、同定されている（「微生物による有機化合物の変換」福井三郎監訳、学会出版センター、pp２１-２６）。これらの細菌類は、工場排水等の芳香族化合物により汚染された環境の浄化にごく一般的に用いられてきている。そして近年、このような酵素が、生体に対し有害でありかつ難分解性である、トリクロロエチレン（以下ＴＣＥ）やジクロロエチレン（以下ＤＣＥ）といった揮発性有機塩素化合物の分解に関与していることがいくつかの研究で明らかにされてきている。例えば、分解対象物がＴＣＥの場合、その分解細菌は芳香族化合物資化性ＴＣＥ分解菌と呼ばれ、トルエンモノオキシゲナーゼを有するAcinetobacter sp.strain G4（Appl.Environ.Microbiol.,52,383（1986）、同53,949（1987）、同54,951（1989）、同56,279（1990）、同57,193（1991））、Pseudomonas mendocina KR-1（Bio/Technol.,7,282（1989））、トルエンジオキシゲナーゼを有するPseudomonas putida F1（Appl.Environ.Microbiol.,54,1703（1988）、同54,2578（1988））、フェノールヒドロキシラーゼを有するPseudomonas putida BH（Wat.Res.,27,9（1993））等が報告されている。

【０００３】このような芳香族化合物分解菌のスクリーニングを行ったり、それらの分解活性を確認したりする際、これまでその指標となる物質として2-ヒドロキシムコン酸セミアルデヒド（以下ＨＭＳ）或いはその誘導体が用いられ、かかる物質の生成の有無で微生物の分解活性を確認していた。ＨＭＳ或いはその誘導体は、芳香核に水酸基を一つ以下有する化合物、具体的にはフェノール、トルエン、クレゾールが、フェノールヒドロキシラーゼ等の酵素によってカテコール核を持つ化合物に酸化された後、カテコール-２,３-オキシゲナーゼによってメタ開裂を受けることによって生成する化合物である（Appl.Environ.Microbiol.,53,949（1987）、同57,1935（1991））。ＨＭＳ或いはその誘導体は黄色を呈するから、かかる物質が生成されたときの色調変化を捉えることにより、微生物のトルエンやフェノールといった化

合物の分解活性を確認することができるので、微生物分解活性の指標として広く用いられてきた。

【０００４】該物質を指標として用いる簡便な方法としては、微生物の有するカテコール分解活性が他の芳香族化合物分解活性の指標になると見做して、液体培地にカテコールを加えるか或いはプレートにカテコール水溶液を散布するかして培養し黄色の呈色を目で確かめるという方法が通常採用されている。しかし、ＨＭＳ或いはその誘導体は微生物によって非常によく分解されてしまうため、黄色を呈している時間は非常に限られたものであり、該呈色を明瞭に確認できる時間が短いので、微生物の分解活性の確認作業上問題があった。また、かかる呈色は微生物の分解活性の有無の指標にはなるが、微生物が高い分解活性を発現する段階とは必ずしも相応していないという問題があった。

【０００５】このような問題を解決するため、Shieldsらはm-トリフルオロメチルフェノール（ＴＦＭＰ）が、カテコール核を生成しメタ開裂をおこした後は、黄色を呈するにもかかわらず微生物分解を非常に受けにくいことを利用して、トルエンやフェノールといった化合物の分解活性の指標物質の前駆物質としてＴＦＭＰを用いている（Appl.Environ.Microbiol.,57,1935（1991））。しかし、この黄色の呈色は、特にプレート上或いは液体培養でも２ｘＹＴ培地やＬＢ培地のような有色培地等では菌の分泌物等の色と識別しにくいことも多いので、微生物の分解活性を的確に確認することが難しく、また、呈色時期と微生物が高い分解活性を発現する段階とが必ずしも相応しておらず、あまり実用的な方法とは言えなかった。

【０００６】

【発明が解決しようとする課題】本発明は、上記従来技術の有する問題点に鑑み、微生物有する芳香族化合物および／または有機塩素化合物分解活性を確認する指標として、呈色物質であるＨＭＳや呈色物質の前駆物質であるＴＦＭＰ以外の特定の物質を用いることにより、明瞭に識別でき、かつ安定しており長時間維持でき、また分解活性の発現段階に相応する呈色を得、もって、微生物の分解活性の有無の確認および微生物が高い分解活性を発現する段階の確認を確実に、かつ簡便に行うことを目的とする。また、本発明の他の目的は、かかる微生物分解活性の確認方法を用いて、複数の微生物から、芳香族化合物および／または有機塩素化合物分解活性を有する微生物を簡便に効率良くスクリーニングすることである。

【０００７】また、本発明の更に他の目的は、かかる微生物分解活性の確認方法を用いて、高い分解活性を発現する段階の微生物を回収精製し、これを用いて芳香族化合物および／または有機塩素化合物により汚染された環境を効率的に浄化することである。

【０００８】

【課題を解決するための手段】かかる目的を達成する本発明は、クレオソートの存在下で微生物を培養して該クレオソートを該微生物により分解し得られる呈色物質の生成を指標として、該微生物の有する芳香族化合物および／または有機塩素化合物分解活性を確認することを特徴とする微生物分解活性確認方法である。クレオソートを呈色物質の前駆物質として用いることにより、赤褐色の呈色が得られ、該呈色は明瞭に識別でき、かつ安定しており長時間維持でき、また分解活性の発現段階に相応しているので、微生物の分解活性の有無の確認および微生物が高い分解活性を発現する段階の確認を確実に、かつ簡便に行うことが可能となる。ここで、有効に呈色を発現させるには、好ましくは、クレオソートの濃度は培地中１０～１０００ppmがよく、また、好ましい対象としては、確認する芳香族化合物分解活性等は置換ベンゼン分解活性または塩素化エチレン分解活性であり、また、微生物としては芳香族化合物を酸化分解できる微生物や芳香族化合物資化揮発性有機塩素化合物分解菌が好ましく、特に、シュードモナス属に属する微生物であって、シュードモナス・セパシアまたはシュードモナス・プチダが好ましい。

【０００９】また、本発明は、芳香族化合物および／または有機塩素化合の存在下で複数の微生物を培養してコロニーを形成させて一種以上の微生物をスクリーニングし、次に、クレオソート含有溶液を該コロニーに接触させて培養し、該クレオソートを分解する微生物を呈色物質の生成を指標としてスクリーニングすることを特徴とする芳香族化合物および／または有機塩素化合物分解活性を有する微生物のスクリーニング方法である。クレオソートを用いた微生物分解活性の確認方法を採用することにより、複数の微生物から、芳香族化合物および／または有機塩素化合物分解活性を有する微生物を簡便に効率良くスクリーニングすることが可能となる。

【００１０】また、本発明は、クレオソートの存在下で微生物を培養して該クレオソートを該微生物により分解し、培養液がクレオソートの分解により呈色するのを確認した後、該培養液から該微生物を回収精製し、この微生物を、芳香族化合物および／または有機塩素化合物により汚染された環境に分散させ該環境を浄化することを特徴とする微生物による環境浄化方法である。クレオソートを用いた微生物分解活性の確認方法を採用することにより、高い分解活性を発現する段階の微生物を回収精製し、これを用いて芳香族化合物および／または有機塩素化合物により汚染された環境を効率的に浄化することが可能となる。また、クレオソートは、芳香族化合物資化性揮発性有機塩素化合物分解菌に対して、有機塩素化合物分解活性を誘導する誘導物質として機能するので、芳香族化合物等の誘導物質が存在しない環境中でも、該環境を浄化、修復することが可能となる。

【００１１】以下、本発明を詳述する。

【００１２】本発明におけるクレオソートは、ブナ材のタール分を蒸留・精製した化合物であり、その成分はグアヤコール、クレゾールが全体の５０～８０％を占め、その他、クレゾール類、メチルクレオソール、キシレノール類等が含有されている。局方として入手できるが、それに限定されるものではなく、クレオソートとしての作用効果を有するものであれば用いることができる。

【００１３】本発明の芳香族化合物および／または有機塩素化合物の微生物分解確認の指標として用いる赤褐色の呈色物質は、クレオソートが微生物によって酸化分解された物質であることは明らかであるが、クレオソートの主成分であるグアヤコールやクレゾールの酸化物ではない。それは、クレオソート液化物の呈色はグアヤコール或いはクレゾール単独の微生物酸化物の呈色とは明かに異なっているからである。

【００１４】ＨＭＳ或いはその誘導体が黄色を呈するのに対し、本発明におけるクレオソートの分解物は赤褐色を呈し、プレート上でも目で見て極めて容易に識別することができる。また、ＨＭＳ或いはその誘導体の呈色が３０℃で４～５時間で消失してしまうのに対し、クレオソートの分解物の呈色は開放系で２４～４８時間程度、密閉系ではほぼ半永久的に消失しない。従って、培養後、菌フェーズが定常期に入った段階でも、分解活性を評価することができる利点がある。また、赤褐色の呈色を確認する手段としては、目視の他、ＵＶ－ＶＩＳ吸光計等の各種光学機器を用いることができる。

【００１５】用いるクレオソートの濃度としては、微生物が培養により分解活性を発現した場合に赤褐色に呈色するに足りる濃度であり、培地に存在する微生物数、微生物の種類、培地の種類、培養形態等により相違するが、概ね、１０～１０００ppm程度、好ましくは５０～２００ｐｐｍ程度がよい。例えば、液体培養系では、菌体10^7 cells／ｍｌ培地（培養後）に対し１０～１０００ppm、好ましくは５０～５００ppm程度がよく、また、スクリーニング用のプレートではプレート当り（１コロニー当り）５０～５００ppm、好ましくは１００～３００ppm程度の水溶液を０．１～１ml（プレート表面積φ９０ｍｍ当り）程度噴霧するとよい。クレオソートの濃度が低過ぎると呈色の度合が低く、活性を確認し難くなり、高過ぎると菌自体の増殖、活性が抑制される。また、クレオソートを培地に添加する時期は、微生物の培養初期から液体培地に添加してもよいし、また、ある程度（Ｏ．Ｄ．で０．５～１．２程度）まで培養した後、別に用意した培地に添加してもよい。クレオソートの添加された培地では微生物の増殖が阻害される可能性があるので、初めに培養培地で一定数になるまで微生物を増殖させ、これを別のクレオソートの添加された培地に移し培養するのが好ましい。また、微生物のスクリーニングでは用いるプレート（寒天培地）上にコロニーが形成された後に水溶液としたクレオソートを噴霧するとよい。

【００１６】本発明で対象とする微生物としては、芳香族化合物をオキシゲナーゼ系の酵素によって酸化分解する微生物であればよく、特に、芳香族化合物資化性揮発性有機塩素化合物分解菌に関しては有機塩素化合物の分解活性が芳香族化合物系の誘導物質によって誘導されるものであれば制限なく利用できる。このような微生物としては、未同定の微生物、単離されていない微生物、共生系の微生物群、単離・同定された微生物が利用できる。同定されている微生物としては、酵母や、シュードモナス属、アシネトバクター属、キサントバクター属等に属する細菌で上記の性質を有するものが利用でき、特にシュードモナスセパシア、シュードモナスプチダ、シュードモナスフルオレセンス、シュードモナスアルギノーザ等の細菌が有効であり、例えばタカサゴシロアリの腸内より単離されたシュードモナスセパシアＫＫ０１株（FERM BP-4235）、フェノール馴養活性汚泥より分離されたシュードモナスプチダＢＨ株（下水道協会誌，Vol.24,No.273,pp27-33（1987）;Wat,Res.,Vol.27,No.1,pp9-13（1993））等を挙げることができる。

【００１７】本発明において、スクリーニングする微生物の分解活性の対象となる、または高い分解活性を確認する対象となる芳香族化合物としては、フェノール、トルエン、o-クレゾール、m-クレゾール、p-クレゾール、バニリン、バニリン酸等の置換ベンゼンを有効なものとして挙げることができる。

【００１８】本発明において、スクリーニングする微生物の分解活性の対象となる、または高い分解活性を確認する対象となる揮発性有機塩素化合物としては、ＴＣＥやＤＣＥ等の塩素化エチレン系の化合物を有効なものとして挙げることができる。

【００１９】これら芳香族化合物や有機塩素化合物は、環境浄化技術、物質変換処理技術等の分野において特に重要な化合物であり、産業上有益なものである。

【００２０】本発明の微生物分解活性確認方法を用いて効率的に芳香族化合物および／または有機塩素化合分解活性を有する微生物をスクリーニングすることができる。スクリーニングでは第一スクリーニングからプレート上に噴霧してもよいし、芳香族化合物を指標として第一スクリーニングをかけ、形成されたコロニーの上にクレオソート含有溶液を噴霧し第二回以降のスクリーニングを行ってもよい。雑多な微生物が混在していると予想される場合は、初めに芳香族化合物および／または有機塩素化合物をマカーとして第一スクリーニングをかけ、次にクレオソートを添加した培地によりスクリーニングをかけるのが効率的であり好ましい。

【００２１】また、本発明の微生物分解活性確認方法を用いて、環境浄化処理等を行う場合は、クレオソートの存在下で微生物を培養して呈色した培養液から微生物を回収精製し、この微生物を、芳香族化合物および／また

は有機塩素化合物により環境修復を目的とした環境に分散すればよい。微生物の分解活性と微生物の増殖菌数とは必ずしも一致しておらず、培養の結果、所定の菌数となったとしても、分解活性が高いとは言えないが、本発明の方法によれば、増殖菌数に拘らず、高い分解活性を発現する段階で微生物を回収することが可能となる。また、クレオソートは、芳香族化合物資化性揮発性有機塩素化合物分解菌に対して、有機塩素化合物分解活性を誘導する誘導物質として機能するので、フェノール等の芳香族化合物誘導物質が存在しない環境中でも、該環境を浄化、修復することが可能となる。

【００２２】

【実施例】以下実施例により本発明をさらに詳細に説明する。なお、各実施例で用いたＭ９培地は下記の組成を有するものである。

【００２３】Ｍ９培地組成（１リットル中）；

Na_2HPO_4	６．２g
KH_2PO_4	３．０g
ＮａＣｌ	０．５g
NH_4Cl	１．０g
水	残部

（ｐＨ７．０、ＢＨ株の場合のみ希硫酸で７．６に調整）すべての芳香族化合物濃度の測定は、培養液の遠心分離上澄の紫外吸収を分光光度計（島津自記分光光度計ＵＶ－３１００Ｓ）で測定することによって行った。　すべてのＴＣＥ或いはＤＣＥ濃度の測定は、ヘッドスペース－ガスクロマトグラフィー法で行なった。即ち、２０ml容バイアル瓶に所定のＴＣＥ濃度になるように調整したＭ９培地を５ml加え、菌液を加えた後、ゴム栓、アルミキャップで密閉し、３０℃で一定時間振盪培養した後に気相０．１mlを採取し、ＦＩＤ検出器によりガスクロマトグラフィー分析（島津ガスクロマトグラムGC－１４Ｂ）を行なった。

［実施例１］

（ＫＫ０１株及びＢＨ株による赤褐色物質の生成）前記ＫＫ０１株及びＢＨ株をそれぞれ別個に、グルタミン酸ナトリウム０．２％を含有したＭ９培地の入った３００ml容坂口フラスコ中で３０℃、１２０rpmの条件で振盪培養し、O.D.が０．８になった時点で、グルタミン酸ナトリウム０．２％、クレオソート１００ppmを含有したＭ９培地５mlの入ったバイアル瓶に該培養液を０．５ml加え、３０℃、１２０rpmの条件で振盪培養した。その結果、ＫＫ０１株では４０時間後に培養液が赤褐色を呈し、ＢＨ株では４６時間後に培養液が赤褐色を呈することが確認できた。なお、この際の菌数はＫＫ０１株では１．８×１０^{7} cells、ＢＨ株では１．６×１０^{7} cellsであった。

【００２４】［実施例２］

（ＫＫ０１株の芳香族化合物分解活性確認）ＫＫ０１株（ＦＥＲＭ　ＢＰ－４２３５）を、グルタミン酸ナトリウム０．２％、クレオソート１００ppmを含有したＭ９培地で３０℃、１２０rpmの条件で振盪培養し、培養液が赤褐色を呈した段階（４１時間後）で菌体を遠心分離で回収し、脱イオン水で洗浄した後、菌数が６．０×１０^{7} cells/mlになるよう脱イオン水に懸濁し、フェノール、トルエン、o－クレゾール、m－クレゾール、p－クレゾール、バニリン、バニリン酸をそれぞれ別個に１００ppm含んだＭ９培地５mlの入った試験管に０．５ml加え、３０℃、１２０rpmの条件で３時間振盪培養し、それぞれの化合物の濃度を測定した。なお、ブランクとしては菌液の代わりに脱イオン水０．５ml加えたものを用いた。各化合物の残留％を表１に示す。表から明らかなようにＫＫ０１株の顕著な芳香族化合物分解活性が認められた。

【００２５】

【表１】

表１　ＫＫ０１株の芳香族化合物分解

化合物	残留％
フェノール	２１
トルエン	４４
o−クレゾール	２０
m−クレゾール	１８
p−クレゾール	２５
バニリン	２１
バニリン酸	２９

［比較例２］実施例２と同様の条件で、培養液が赤褐色を呈する前の段階（２４時間後）で菌体を遠心分離で回収し、脱イオン水で洗浄した後、菌数が実施例２と同様になるよう調整して加え、それぞれの化合物の濃度を測定した。各化合物の残留％を表２に示す。

【００２６】

【表２】

表２　ＫＫ０１株の芳香族化合物分解

化合物	残留％
フェノール	９５
トルエン	１０１
o−クレゾール	９８
m−クレゾール	９６
p−クレゾール	１００
バニリン	９９
バニリン酸	９６

以上のようにＫＫ０１株のクレオソート培養液が赤褐色を呈した段階でフェノール、トルエン、o−クレゾール、m−クレゾール、p−クレゾール、バニリン、バニリン酸の分解活性が高まっていることが示された。

【００２７】［実施例３］

（ＢＨ株の芳香族化合物分解活性確認）実施例２と同様の操作をＢＨ株（大阪大学工学部環境工学課　藤田正憲教授より入手）においても行い、培養液が赤褐色を呈した段階（４８時間後）にフェノール、トルエン、カテコールの分解を測定した。各化合物の残留％を表３に示す。表から明らかなようにＢＨ株の顕著な芳香族化合物分解活性が認められた。

【００２８】

【表３】

表3　BH株の芳香族化合物分解

化合物	残留％
フェノール	21
トルエン	44
カテコール	20

［比較例3］実施例2と同様の条件で、培養液が赤褐色を呈する前の段階（28時間後）で菌体を遠心分離で回収し、脱イオン水で洗浄した後、菌数が実施例2と同様になるよう調整して加え、それぞれの化合物の濃度を測定した。各化合物の残留％を表4に示す。

【0029】

【表4】

表4　BH株の芳香族化合物分解

化合物	残留％
フェノール	96
トルエン	99
カテコール	92

以上のようにBH株のクレオソート培養液が赤褐色を呈した段階でフェノール、トルエン、カテコールの分解活性が高まっていることが示された。

【0030】［実施例4］

（KK01株のTCE及びDCE分解活性確認）KK01株を、グルタミン酸ナトリウム0.2％、クレオソート100ppmを含有したM9培地で30℃、120rpmの条件で振盪培養し、培養液が赤褐色を呈した段階（41時間後）で菌体を遠心分離で回収し、脱イオン水で洗浄した後、菌数が6.0×10^7 cells/mlになるよう脱イオン水に懸濁し、TCE、cis-1,2-ジクロロエチレン（c-DCE）、trans-1,2-ジクロロエチレン（t-DCE）をそれぞれ別個に5ppm含んだMP培地5mlの入ったバイアル瓶に0.5ml加え、30℃、120rpmの条件で3時間振盪培養し、それぞれの化合物の濃度を測定した。なお、ブランクとしては菌液の代わりに脱イオン水0.5ml加えたものを用いた。各化合物の残留％を表5に示す。表から明らかなようにKK01株の顕著なTCE及びDCE分解活性が認められた。

【0031】

【表5】

表5　KK01株のTCE及びDCE分解

化合物	残留％
TCE	12
c-DCE	22
t-DCE	50

［比較例4］実施例4と同様の条件で、培養液が赤褐色を呈する前の段階（24時間後）で菌体を遠心分離で回収し、脱イオン水で洗浄した後、菌数が実施例4と同様になるように調整して加え、それぞれの化合物の濃度を測定した。各化合物の残留％を表6に示す。

【0032】

【表6】

表6　ＫＫ０１株のＴＣＥ及びＤＣＥ分解

化合物	残留％
ＴＣＥ	９９
c-ＤＣＥ	１００
t-ＤＣＥ	９９

以上のようにＫＫ０１株のクレオソート培養液が赤褐色を呈した段階でＴＣＥ、c-ＤＣＥ、t-ＤＣＥの分解活性が高まっていることが示された。

【００３３】［実施例５］

（ＢＨ株のＴＣＥ分解活性確認）実施例４と同様の操作をＢＨ株においても行い、培養液が赤褐色を呈した段階（４８時間後）でＴＣＥの分解を測定したところ、残留％は１５％であった。

【００３４】［比較例５］実施例５と同様の要領で、培養液が赤褐色を呈する前の段階（２８時間後）で菌体を遠心分離で回収し、脱イオン水で洗浄した後、菌数が実施例５と同様になるよう調整して加え、ＴＣＥの分解を測定したところ、残留％は９８％であった。

【００３５】以上のようにＢＨ株のクレオソート培養液が赤褐色を呈した段階でＴＣＥの分解活性が高まっていることが示された。

【００３６】［実施例６］

（フェノール分解菌のスクリーニング）土壌中には通常多くのフェノール分解菌が棲息していることが知られている。そこで、武蔵野ロームに分類されるごく一般的な関東ローム層から、ローム土１０gを採取し、滅菌水１００mlを加えてブレンダ処理を行って、その懸濁液を、グルタミン酸ナトリウム０．２％、フェノール１００ppmを含むＭ９寒天培地に塗布した。この寒天培地を３０℃で３日間培養すると、７種類の異なるコロニーが確認された。しかし、培地中には炭素源としてフェノールの他にグルタミン酸ナトリウムを含んでいるため、この7種類の土着菌がすべてフェノール分解菌であるとは限らない。そこで、2次スクリーニングとしてクレオソートの１００ppm水溶液をプレート上に噴霧し、さらに３０℃で２日間培養した。その結果、７種類のコロニーのうち５種類の周辺に赤褐色の呈色が見られ（これらをＡ、Ｂ、Ｃ、Ｄ、Ｅとする）、２種類については無色のまま（これらをＦ、Ｇとする）であった。これらの７種類のコロニーを分離し、グルタミン酸ナトリウム０．２％、フェノール１０００ppmを含む同組成のＭ９液体培地５mlを入れた試験官で３０℃、１２０rpmの条件で振盪培養し、O.D.が０．５になった時点でフェノールを１００ppmになるように加え、３日間同条件で振盪培養し、フェノールの濃度を測定した。各土着菌におけるフェノールの残留％を表7に示す。

【００３７】

【表7】

表7 各土着菌のフェノール分解

菌	残留フェノール%
A	20.0
B	15.3
C	22.5
D	1.2
E	0.5
F	99.5
G	98.0

以上のようにクレオソート水溶液が赤褐色を呈したコロニーにのみフェノール分解能が認められた。

【0038】

【発明効果】以上説明したように、本発明によれば、これまで煩雑で不明瞭であった芳香族資化菌及び芳香族化合物資化性揮発性有機塩素化合物分解菌スクリーニング方法或いは分解活性確認方法が飛躍的に改善され、より確実に該スクリーニング或いは該分解活性確認が行えるようになった。即ち、クレオソートを呈色物質の前駆物質として用いることにより、赤褐色の呈色が得られ、該呈色は明瞭に識別でき、かつ安定しており長時間維持でき、また分解活性の発現段階に相応しているので、微生物の分解活性の有無の確認および微生物が高い分解活性を発現する段階の確認を確実に、かつ簡便に行うことが可能となる。

【0039】また、クレオソートを用いた微生物分解活性の確認方法を採用することにより、複数の微生物から、芳香族化合物および／または有機塩素化合物分解活性を有する微生物を簡便に効率良くスクリーニングすることが可能となる。また、クレオソートを用いた微生物分解活性の確認方法を採用することにより、高い分解活性を発現する段階の微生物を回収精製し、これを用いて芳香族化合物および／または有機塩素化合物により汚染された環境を効率的に浄化することが可能となる。また、クレオソートは、芳香族化合物資化性揮発性有機塩素化合物分解菌に対して、有機塩素化合物分解活性を誘導する誘導物質として機能するので、芳香族化合物等の誘導物質が存在しない環境中でも、該環境を浄化、修復することが可能となる。

フロントページの続き

(51)Int.Cl.^{6}	識別記号	庁内整理番号	FI	技術表示箇所
C12R 1:40)				
(C12Q 1/04				
C12R 1:38)				
(C12N 1/00				
C12R 1:01)				
(C12N 1/20				
C12R 1:38)				

6/7/1 (Item 1 from file: 5)
DIALOG(R)File 5:Biosis Previews(R)

12249160 BIOSIS NO.: 200000002662
Construction of recombinants Pseudomonas **putida** BO14 and Escherichia coli QEFCA8 for ferulic acid biotransformation to **vanillin**.
AUTHOR: Okeke Benedict C(a); Venturi Vittorio
AUTHOR ADDRESS: (a)Laboratory of Microbial Engineering and Technology, Department of Bioprocessing, Gifu University, 1-1 Yanagido, Gifu, 501-1193**Japan
JOURNAL: Journal of Bioscience and Bioengineering 88 (1):p103-106 July, 1999
ISSN: 1389-1723
DOCUMENT TYPE: Article
RECORD TYPE: Abstract
LANGUAGE: English
SUMMARY LANGUAGE: English

ABSTRACT: Recombinants Pseudomonas **putida** BO14 and Escherichia coli QEFCA8 capable of ferulic acid biotransformation to **vanillin** were constructed using homologous recombination and a PCR based cloning strategy, respectively. In the liquid culture of P. **putida** BO14, 26.81+-2.30 mug **vanillin** ml-1 of culture filtrate was detected. In the case of recombinant E. coli QEFCA8, 19.37+-1.95 mug **vanillin** ml-1 of culture filtrate was detected. Results indicate that the strains could be useful for the biotechnological production of **vanillin**, a very important flavoring substance.

6/7/2 (Item 2 from file: 5)
DIALOG(R)File 5:Biosis Previews(R)

11983295 BIOSIS NO.: 199900263814
Towards a high-yield bioconversion of ferulic acid to **vanillin**.
AUTHOR: Muheim A(a); Lerch K
AUTHOR ADDRESS: (a)Givaudan Roure Research Ltd., Ueberlandstr. 138, CH-8600, Dubendorf**Switzerland
JOURNAL: Applied Microbiology and Biotechnology 51 (4):p456-461 April, 1999
ISSN: 0175-7598
DOCUMENT TYPE: Article
RECORD TYPE: Abstract
LANGUAGE: English
SUMMARY LANGUAGE: English

ABSTRACT: Natural **vanillin** is of high interest in the flavor market. Microbial routes to **vanillin** have so far not been economical as the medium concentrations achieved have been well below 1 g l-1. We have now screened microbial isolates from nature and known strains for their ability to convert eugenol or ferulic acid into **vanillin**. Ferulic acid, in contrast to the rather toxic eugenol, was found to be an excellent precursor for the conversion to **vanillin**, as doses of several g l-1 could be fed. One of the isolated microbes, later identified as Pseudomonas **putida**, very efficiently converted ferulic acid to **vanillic** acid. As **vanillin** was oxidized faster than ferulic acid, accumulation of **vanillin** as an intermediate was not observed. A completely different metabolic flux was observed with Streptomyces setonii. During the metabolism of ferulic acid, this strain accumulated **vanillic** acid only to a level of around 200 mg l-1 and then started to accumulate **vanillin** as the principal metabolic overflow product. In shake-flask experiments, **vanillin** concentrations of up to 6.4 g l-1 were achieved with a

molar yield of 68%. This high level now forms the basis for an economical microbial production of **vanillin** that can be used for flavoring purposes.

6/7/3 (Item 3 from file: 5)
DIALOG(R)File 5:Biosis Previews(R)
(c) 2002 BIOSIS. All rts. reserv.

11474848 BIOSIS NO.: 199800256180
Genetics of ferulic acid bioconversion to protocatechuric acid in plant-growth-promoting Pseudomonas **putida** WCS358.
AUTHOR: Venturi Vittorio(a); Zennaro Francesca; Degrassi Giuliano; Okeke Benedict C; Bruschi Carlo V
AUTHOR ADDRESS: (a)Bacteriol. Group, Int. Cent. Genet. Eng. Biotechnol., Area Science Park, Padriciano 99, 34012 Tr**Italy
JOURNAL: Microbiology (Reading) 144 (4):p965-973 April, 1998
ISSN: 1350-0872
DOCUMENT TYPE: Article
RECORD TYPE: Abstract
LANGUAGE: English

ABSTRACT: Transposon Tn5 genomic mutants of plant-growth-promoting Pseudomonas **putida** strain WCS358 have been isolated which no longer utilize ferulic and coumaric acids as sole sources of carbon and energy. Genetic studies confirmed previous biochemical data showing that ferulic acid is degraded via **vanillic** acid, and coumaric acid via hydroxybenzoic acid. The genes involved in these enzymic steps were cloned and characterized. Two proteins designated Fca (26.5 kDa) and Vdh (50.3 kDa) were identified as responsible for the conversion of ferulic acid to **vanillic** acid; the proteins are encoded by the fca and vdh genes which are organized in an operon structure in the chromosome. The Vdh protein is 69% identical at the amino acid level to the Vdh protein recently identified in Pseudomonas sp. strain HR199 and converts **vanillin** to **vanillic** acid. Homology studies revealed that the Vdh proteins exhibited significant identity to aldehyde dehydrogenases from different organisms whereas Fca belonged to the enoyl-CoA hydratase family of proteins. Two proteins, designated VanA (39.9 kDa) and VanB (34.3 kDa), encoded by two genes, vanA and vanB, are organized in an operon in the chromosome. They were found to be responsible for the demethylation of **vanillic** acid to protocatechuic acid. The VanA proteins showed no homology to any other known protein, while VanB belonged to the ferredoxin family of proteins. This two-component enzyme system demethylated another phenolic monomer, veratric acid, thus indicating broad specificity. Studies of the regulation of the vanAB operon demonstrated that the genes were induced by the substrate, **vanillic** acid; however, the strongest induction was observed when cells were grown in the presence of the product of the reaction, protocatechuic acid.

6/7/11 (Item 1 from file: 73)
DIALOG(R)File 73:EMBASE
(c) 2002 Elsevier Science B.V. All rts. reserv.

06048725 EMBASE No: 1995079032
Degradation of methoxylated aromatic acids by Pseudomonas **putida**
Turner J.E.; Allison N.
Microbial Antigens Department, CAMR, Porton Down,Salisbury, Wilts SP4 0JG United Kingdom
Journal of Applied Bacteriology (J. APPL. BACTERIOL.) (United Kingdom)
1995, 78/2 (125-133)
CODEN: JABAA ISSN: 0021-8847
DOCUMENT TYPE: Journal; Article

LANGUAGE: ENGLISH   SUMMARY LANGUAGE: ENGLISH

A newly-isolated strain of Pseudomonas **putida** (HVA-1) utilized homovanillic acid as sole carbon and energy source. Homovanillate-grown bacteria oxidized homovanillate and homoprotocatechuate but monohydroxylated and other methoxylated phenylacetic acids were oxidized poorly; methoxy-substituted benzoates were not oxidized. Extracts of homovanillate-grown cells contained homoprotocatechuate 2,3-dioxygenase but the primary homovanillate-degrading enzyme could not be detected. No other methoxylated phenylacetic acid supported growth of the organism but vanillate was utilized as a carbon and energy source. When homovanillate-grown cells were used to inoculate media containing vanillate a 26 h lag period occurred before growth commenced. Vanillate-grown bacteria oxidized vanillate and protocatechuate but no significant oxygen uptake was obtained with homovanillate and other phenylacetic acid derivatives. Analysis of pathway intermediates revealed that homovanillate-grown bacteria produced homoprotocatechuate, formaldehyde and the ring-cleavage product 5-carboxymethyl 2-hydroxymuconic semialdehyde (CHMS) when incubated with homovanillate but monohydroxylated or monomethoxylated phenylacetic acids were not detected. These results suggest that homovanillate is degraded directly to the ring-cleavage substrate homoprotocatechuate by an unstable but highly specific demethylase and then undergoes extradiol cleavage to CHMS. It would also appear that the uptake/degradatory pathways for homovanillate and vanillate in this organism are entirely separate and independently controlled. If stabilization of the homovanillate demethylase can be achieved, there is potential for exploiting the substrate specificity of this enzyme in both medical diagnosis and in the paper industry.

6/7/12   (Item 1 from file: 94)
DIALOG(R)File 94:JICST-EPlus
(c)2002 Japan Science and Tech Corp(JST). All rts. reserv.

00912000   JICST ACCESSION NUMBER: 89A0330313 FILE SEGMENT: JICST-E
Cloning and expression of Pseudomonas paucimobilis SYK-6 gene involved in
    the degradation of vanillate in P. **putida**. I.
NISHIKAWA S (1); MOROHOSHI N (1); HARAGUCHI T (1); KATAYAMA Y (2); YAMASAKI
    M (3)
(1) Tokyo Univ. Agriculture and Technology, Tokyo, JPN; (2) Kobe Steel
    Ltd., Kobe, JPN; (3) Univ. Tokyo, Tokyo, JPN
Mokuzai Gakkaishi(Journal of the Japan Wood Research Society), 1989,
    VOL.35,NO.2, PAGE.158-163, FIG.6, REF.11
JOURNAL NUMBER: F0852AAU   ISSN NO: 0021-4795   CODEN: MKZGA
UNIVERSAL DECIMAL CLASSIFICATION: 575.116.4
LANGUAGE: English   COUNTRY OF PUBLICATION: Japan
DOCUMENT TYPE: Journal
ARTICLE TYPE: Original paper
MEDIA TYPE: Printed Publication
ABSTRACT: Pseudomonas paucimobilis SYK-6 grows on a medium containing lignin-related compounds as a sole carbon source. We succeeded in isolating the genes involved in the degradation of **vanillic** acid and protocatechuic acid from the genomic library of the bacteria constructed with the Escherichia coli-Pseudomonas shuttle vector, pKT230 (Kmr). Plasmid pVA01 contained the 10.5 kilobase pair EcoRI insert. The transformed cells of P. **putida** PpY 1100 by the plasmid assimilated **vanillic** acid as sole carbon source and were kanamycin resistant. A restriction map of the insert was constructed.(author abst.)

6/7/15   (Item 1 from file: 357)
DIALOG(R)File 357:Derwent Biotech Res.
(c) 2002 Thomson Derwent & ISI. All rts. reserv.

0259799 DBR Accession No.: 2000-14289 PATENT
Converting styrene-containing composition into a second composition containing e.g. benzaldehyde derivative for use as flavor agents, by treating first composition with microorganisms under predetermined conditions - vanilla production using Pseudomonas sp., Rhodotorula sp., Penicillium sp., Aspergillus sp., Zygorrhyncus sp., Micromucor sp., Brevundimonas sp. and Trichoderma sp.
AUTHOR: Cheetham P S J; Gradley M L; Sime J T

inventors

CORPORATE SOURCE: Ashford, UK.
PATENT ASSIGNEE: Zylepsis 2000
PATENT NUMBER: WO 200050622 PATENT DATE: 20000831 WPI ACCESSION NO.: 2000-558401 (2051)
PRIORITY APPLIC. NO.: GB 994251 APPLIC. DATE: 19990224
NATIONAL APPLIC. NO.: WO 2000GB654 APPLIC. DATE: 20000224
LANGUAGE: English
ABSTRACT: A method providing an inter alia method of converting a first composition at least one or more species into a second species using microorganisms, is new. Also claimed are: obtaining a strain of microorganism by screening a multiplicity of colonies by means of a reagent suitable for detecting aldehydes; Pseudomonas **putida** NCIMB40988, Rhodotorula glutinis IMI 379896, Penicillium chrysogenum IMI379900, Aspergillus flavus IMI379895, Aspergillus niger IMI379904, Zygorrhyncus moelleri IMI379899, Pseudomonas **putida** NCIMB 40988, Aspergillus fumigatus IMI379902 and Micromucor isobellinus IMI379893 and their mutants capable of converting **vanillic** acid into **vanillin** and Brevundimonas vesicularis NCIMB40987 and its mutants capable of converting vanillyl alcohol into **vanillian** and Trichoderma koningii IMI379903 and mutants capable of converting 4-hydroxylbenzoic acid into 4-hydroxybenzaldehyde; further defined microbial conversions. The vanilla composition is used as a flavor component in food, or an aroma emitting component for use in cosmetics etc.. Preferred compositions and mediums are given. (62pp)

6/7/19 (Item 5 from file: 357)
DIALOG(R)File 357:Derwent Biotech Res.
(c) 2002 Thomson Derwent & ISI. All rts. reserv.

0138963 DBR Accession No.: 92-11455 PATENT
High-yield **vanillin** production from ferulic acid - or analogs using Pseudomonas **putida**, Aspergillus niger, Rhodotorula glutinis or Corynebacterium glutamicum
PATENT ASSIGNEE: Kraft-Gen.Foods 1992
PATENT NUMBER: US 5128253 PATENT DATE: 920707 WPI ACCESSION NO.: 92-249426 (9230)
PRIORITY APPLIC. NO.: US 708543 APPLIC. DATE: 910531
NATIONAL APPLIC. NO.: US 708543 APPLIC. DATE: 910531
LANGUAGE: English
ABSTRACT: Production of **vanillin** (I) comprises mixing an aq. solution of 0.025-1% **vanillin** precursor (II) with a ferulic acid degrading microorganism in the presence of a sulfhydryl compound (III). The reaction medium also contains an assimilable C-source, preferably glucose, fructose, maltose and/or sucrose. (II) is ferulic acid, eugenol, coniferyl alcohol or 4-vinyl guaiacol. (III) is dithiothreitol, dithioerythritol, glutathione, cysteine, HS-CH2-(CHOH)n -CH2-SH (where n = 4), 2-mercaptoethanol, mercaptoacetic acid and/or S-containing amino acids. The microorganism may be Pseudomonas **putida** ATCC 55180, Aspergillus niger ATCC 11414, Rhodotorula glutinis ATCC 74056 or Corynebacterium glutamicum ATCC 15032. Bioconversion is carried out at pH 3-7 and 20-40 deg, with 1-100 mM (III). The cells are in the stationary phase and reducing conditions are maintained. The culture mixture may include a complexing agent, adsorbent or extractant selective for (I). Inclusion of (III) in the

conversion mixture significantly increases (I) production. The bioconversion process also produces other compounds which impart flavor to (I). (7pp)

?